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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 5/06, 5/08, C07K 19/00, 17/06, A61K 35/14, 35/28, A61P 43/00 // A61L 15/00, C12N 15/62	A2	(11) International Publication Number: WO 00/50570 (43) International Publication Date: 31 August 2000 (31.08.00)
(21) International Application Number: PCT/CA00/00162 (22) International Filing Date: 23 February 2000 (23.02.00) (30) Priority Data: 09/256,499 23 February 1999 (23.02.99) US (71) Applicant: UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; University Of Industry Of Liaison Office, 214 Health Sciences Mall, IRC 331, Vancouver, British Columbia V6T 1Z3 (CA). (72) Inventors: KILBURN, Douglas, G.; 3728 West 29th Avenue, Vancouver, British Columbia V6S 1T4 (CA). JERVIS, Eric; #4-415 Kingscourt Drive, Waterloo, Ontario N2K 3R4 (CA). DOHENY, James, G.; #202-4691 West 10th Avenue, Vancouver, British Columbia V6R 2J3 (CA). HAYNES, Charles, A.; #103 - 2730 Acadia Road, Vancouver, British Columbia V6T 1R9 (CA). (74) Agent: SWABEY OGILVY RENAULT; Suite 1600, 1981 McGill College Avenue, Montreal, Quebec H3A 2Y3 (CA).	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: COMPOSITIONS AND METHODS FOR MODULATING GROWTH OR DIFFERENTIATION OF GROWTH-FACTOR DEPENDENT CELLS		
(57) Abstract This invention relates to compositions and methods for modifying growth or differentiation of growth-factor dependent cells, using fusion proteins composed of a growth factor and a binding domain derived from a polysaccharidase linked diffusively to a solid support. The invention is exemplified by the use of a fusion protein that includes stem cell growth factor linked to a binding domain derived from a bacterial cellulase bound to a solid support to modify growth and/or differentiation of hematopoietic cells.		

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COMPOSITIONS AND METHODS FOR MODULATING GROWTH OR DIFFERENTIATION OF GROWTH-FACTOR DEPENDENT CELLS

INTRODUCTION

5

Technical Field

This invention relates to compositions and methods for modifying growth or differentiation of growth-factor dependent cells, using fusion proteins composed of a growth factor and a binding domain derived from a polysaccharidase linked
10 diffusively to a solid support. The invention is exemplified by the use of a fusion protein that includes stem cell growth factor linked to a binding domain derived from a bacterial cellulase bound to a solid support to modify growth and/or differentiation of hematopoietic cells.

15 Background

A major objective in bioprocess engineering is the development of cell culture methods which provide the environmental conditions necessary for maximum cell densities and specific cell derived product recovery. *In vivo*, growth factors for various target cells often are associated with the extracellular matrix (ECM). The
20 major extracellular matrix components are proteoglycans of which there are four major forms: heparin sulfate, chondritin sulfate, keratan sulfate and hyaluronic acid. The binding properties of these ECM proteins are primarily determined by the form of the glycosaminoglycan carried on the proteoglycan. These molecules appear to protect the growth factors from proteolytic degradation and are thought to be an
25 important reservoir of growth factors in the ECM. Proteoglycans are an abundant and ubiquitous tissue component and are likely to capture a majority of intercellular growth factors for which they have affinity. It has been suggested that the combined action of diffusable factors and nondiffusable matrix signals may be an important mechanism for localizing responses of cells to a cytokine that is widely distributed
30 within an organism. Several cytokines, including basic fibroblast growth factor (bFGF), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3), have been shown to function when bound to proteoglycans of the ECM.

Of particular interest for culturing *in vitro* are cells of the hematopoietic system. The culturing and expansion of bone marrow cells *in vitro* has required the use of irradiated preformed stromal-cell feeder layers. Dexter *et al.* ((1993) *Nature*, 360:709-710) demonstrated that this technique maintained cell populations which
5 were able to regenerate hematopoiesis in irradiated mice. Since these initial studies, various combinations of cytokines added to growth medium have been reported to effectively replace the stromal layer for maintenance of human hematopoiesis in culture. As an example, Matrigel, a commercially available artificial ECM, has been used to immobilize IL-3 and GM-CSF for growing factor dependent IL-3 and GM-
10 CSF cell lines. Immobilized heparin sulfate can be used to replace the Matrigel. In addition, several factors or combinations of factors can be substituted for one another to obtain similar target cell responses. As an example, *Steel* factor (SLF), SCF or granulocyte colony stimulating factor (G-CSF) plus IL-3 can be substituted for feeder layers in supporting LTC-IC maintenance, although relatively high concentrations of
15 the factors are required. However, surface expression of IL-3 receptors is down-regulated in response to high concentrations of IL-3. Genetically engineered stromal cells transfected to produce G-CSF, GM-CSF and IL-3 alone or in combination also have been shown to enhance the maintenance of LTC-IC cultures significantly.

The *in vitro* expansion of T-cells for adaptive immunotherapy has achieved
20 tumor regression in some patients with advanced cancer. With this technique, T-cells are removed from a cancer patient and expanded in culture. Once a sufficient number of cells have been produced, they are reintroduced into the patient. A major obstacle in the expansion of activated T-cells *in vitro* has been the complexity and the expense of processing cultures using conventional tissue culture procedures. Furthermore,
25 when high concentrations of growth factors such as IL-2 are added to IL-2 dependent cells, receptors on the cell surface for IL-2 are down regulated as the IL-2 concentration increases. To counteract this problem, liposomes have been used to increase the IL-2 dependent proliferation of CTLL cells. Use of 20 nM lipids with recombinant human IL-2 greatly increased cell proliferation; proliferation was the
30 most pronounced at low dosages of IL-2. At saturating dosages of IL-2, the liposomes had no significant effect. Additionally, lipid concentrations above 120 nM were found to inhibit proliferation. Thus, another hematopoietic cell for which efficient *ex*

vivo expansion techniques are required is the T-lymphocyte. A dialysis perfusion bioreactor for the expansion of lymphokine-activated killer T-cells for adoptive immunotherapy also has been used. The doubling times, surface molecule phenotype and cytolytic activities were similar for cells produced in the perfusion bioreactor or
5 in standard tissue-culture plates.

SLF, as well as a number of other growth factors, can act as attachment factors when adsorbed non-specifically to plastic, and have been reported to stimulate the proliferation of primitive progenitor cells (Long *et al.* (1992) *J. Clin. Invest.* 90:251-255). Such methods of immobilization, however, do not ensure that the growth factor
10 is presented in the correct orientation to activate the growth-factor receptor. A polar affinity tag may facilitate attachment in the correct orientation but most of the commonly used affinity tags, such as hexahistidine (Smith *et al.* (1988) *J. Biol. Chem.* 263:7211-7215), Streptavidin (Kasher *et al.* (1986) *Mol. Cell. Biol.* 6:3117-3127), or GST (Smith and Johnson (1988) *Gene* 67:31-40) rely on matrices which could
15 interfere with the *in vitro* culture conditions. Therefore, it is of interest to develop a system for the *in vitro* cultivation of cells for therapeutic reinfusion to the body which can provide for continuous maintenance and/or expansion of stem cells and other growth-factor dependent cell populations. Of particular interest is the development of an artificial surface which mimics the stromal membrane for presentation of
20 regulatory factors could have wide applications in cell culture, gene therapy, and analysis of factor-target cell interactions.

Relevant Literature

The observed surface mobility of adsorbed CBD was previously reported to be
25 based on a two-dimensional extension of the steric-exclusion theory of McGhee and von Hippel [21], for CBD_{Cex} adsorption to crystalline cellulose [11]. Steric-exclusion theory assumes that adsorbed protein molecules are static, which is not supported by the FRAP results reported herein. A large fraction of bound CBD molecules are mobile and can therefore redistribute on the surface so that binding-site exclusion
30 does not occur and close packing of adsorbed CBDs is possible. Therefore it is now concluded that the previous model is incorrect and that a two-site adsorption model is more appropriate to explain CBD-cellulose adsorption data.

Artificial matrices, such as surface hydrolyzed poly(methyl methacrylate) films (Ito *et al.*, (1992) *Biomaterials* 13:789-794), or glass beads (Ito *et al.* (1992) *Biotechnology and Bioengineering* 40:1271-1276) with adsorbed or covalently attached growth factors have been used to replace extracellular matrices (ECMs) for growing cells *in vitro*. Immobilized insulin (Liu *et al.* (1992) *Biomaterials* 13:50-58 and Zheng *et al* (1994) *Biomaterials* 15:963-968), transferrin (Liu *et al.* (1993) *International J. Biological Macromolecules* 12:449-453, Liu *et al* (1993) *Int. J. Biol. Macromol* 15:221-226), , and insulin, transferrin, and collagen (Ito *et al.* (1991) *Biomaterials* 12:449-453) have been used with varying degrees of success.

Compositions and methods relating to cellulose and 1,4- -glycan binding domains are described in the following US patents: USPN 5,340,731; USPN 5,202,247; and USPN 5,137,819. Also see PCT application PCT/US94/04132, which describes cellulose binding domain proteins ("scaffolding proteins"). Also see U.S. Patents 5,856,201; 5,837,814; 5,738,984; 5,719,044; 5,670,623; and 5,496,934. Also see Guarna, Jervis, Doheny, Orchansky, Warren and Kilburn (1997) (Lake Tahoe, NV) Cytokine 9: 927; Jervis, Doheny, Guarna, Kilburn and Haynes (October 1997) 47th CSCHE Conference October, Edmonton, Alberta; Jervis, Doheny, Guarna, and Kilburn (April 1997) ACS Meeting, San Francisco, CA; Kilburn, Doheny, Guarna, Humphries, and Warren (January 1996) Cell Culture Engineering V, San Diego, CA.; Jervis *et al.*, (1996) ACS Spring Meeting, New Orleans, LA.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for supplying surface localized growth factors to growth-factor dependent cells through the use of diffusibly immobilized growth factors to be presented in the proper orientation to a cellular receptor for the growth factor to activate the receptor. The compositions include a soluble or insoluble matrix to which a growth-factor conjugate comprising a growth factor and a substrate binding region derived from a polysaccharidase are bound. The substrate binding region preferably is essentially lacking in hydrolytic activity of the polysaccharidase. Also included are compositions wherein cells having a growth-factor receptor are bound to the matrix; and soluble growth factors which are derived from membrane-anchored precursors which include an extracellular region

containing the growth-factor domain, a hydrophobic domain and a small cytoplasmic domain. Methods of expansion of growth-factor dependent cells also are provided. The methods involve growing cells with a cell surface receptor for the growth factor in contact with the growth-factor conjugate. To obtain a population of cells enriched in growth-factor dependent cells, a plurality of cells are contacted with the growth-factor conjugate and any cells lacking the cell surface receptor are then removed. Optionally, the cells can be removed from the conjugate or the substrate. The methods are useful for *in vitro* cultivation of growth-factor dependent cells including, hematopoietic cells such as stem cells and megakaryocytic, other bone marrow and blood cells, nerve cells, and T cells. The matrices with cells attached can be used as tissue prostheses. The growth-factor conjugates can be used for *in vitro* stimulation of cells for promoting *ex vivo* growth of cells prior to reengraftment, for enhancing healing of a wound by contacting the wound with the growth-factor conjugate that is optionally bound to a wound covering.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A through 1D show the SLF-CBD fusion protein and controls. The extracellular domain of murine steel factor (Fig. 1A) was used to replace the catalytic domain of the exoglucanase Cex (Fig. 1B) to create the fusion protein SLF-CBD (Fig. 1C). The recombinant extra cellular domain of murine steel factor was used as a positive control (Fig. 1D) and CBD_{cex} was used as a negative control (not illustrated). The fusion junctions are depicted for SLF-CBD under the diagram of the SLF-CBD fusion protein (Fig. 1C). Underlined amino acids are the native SLF sequence. The N-terminal fusion junction has the following amino acid sequence denoted by standard single letter code for amino acids: ASHHHHHHIEGRARKEI (SEQ ID No: 1). The C-terminal fusion junction has the following sequence: PPVASIEGRTSQAFGAS (SEQ ID No: 2). Both fusion junctions contain the cleavable IEGR (SEQ ID No: 3) sequence from Factor Xa.

Figures 2A and 2B show a graphic representation of the pTugA expression vector from which the expression vector used for high level expression of SLF-CBD fusion proteins in *Escherichia coli* was derived. Use of pTugA results in high level inducible transcription, enhanced RNA translation, portability, high copy number,

stability and versatility. The pTug vectors contain the mutant pMB1 *ori* derived from pUC13 to enhance copy number (Minton *et al. Focus* (1988) 10:56), a strong and highly inducible (by IPTG) *tac* promoter (P_{tac}) which is strongly repressed by LacIq. The *lacIq* allele is incorporated in the pTug vector to maintain a constant ratio of P_{tac} to LacIq, ensuring adequate levels of repressor irrespective of the *E. coli* host. The *gene10* translational enhancer (Olins *et al. Gene* (1988) 73:227) is also incorporated in the pTug vector. The leader sequence of the endoglucanase A (CenA) from *C. fimi* was incorporated in the vector to allow recovery of a recombinant polypeptide from *E. coli* supernatants. Fig. 2A shows (SEQ ID No: 4) the nucleotide and (SEQ ID No: 5) the encoded amino acid sequence of the *NcoI* - *HindIII* region as well as the nucleotide sequence of the region upstream of the *NcoI* site, including the gene 10 translational enhancer ("g10") and the CenA leader sequence ("leader"). Fig. 2B shows the pTugA vector map.

Figures 3A and 3B show a graphic representation of the pTugAS. Fig. 3A shows (SEQ ID No: 6) the nucleotide and (SEQ ID No: 7) the encoded amino acid sequence of the *SacI*-*HindIII* region as well as the nucleotide sequence of the region upstream of the *SacI* site. Fig. 3B shows the pTugAS vector map.

Figures 4A and 4B show purification of the SLF-CBD fusion protein. SLF-CBD can be purified using either of its two affinity tags. Fig. 4A shows purification of SLF-CBD with cellulose: *lane 1*, markers; *lane 2*, conditioned growth medium; *lane 3*, fusion protein recovered from 5 ml of conditioned growth medium by absorption to 5 mg of Avicel; *lane 4*, periplasmic extract; *lane 5*, fusion protein extracted directly from 1 ml of periplasmic extract by 5 mg of Avicel. Fig. 4B shows purification of SLF-CBD with nickel Sepharose using the hexahistidine tag: *lane 1*, markers; *lane 2*, fusion protein elution peak.

Figure 5 shows SDS-PAGE and western blotting analysis of SLF-CBD. The identity of the purified fusion protein was confirmed by western blot analysis. Fig. 5A shows SDS-PAGE of SLF-CBD: (Coomassie blue stained) *lane 1*, markers; *lane 2*, prestained markers; *lane 3*, 1 μ g purified SLF-CBD; *lane 4*, 20 ng SLF-CBD cut by Factor Xa (not visible on gel); *lane 5*, 10 ng recombinant control SLF (not visible on gel, dark band is BSA); *lane 6*, 500 ng CND_{ext}; *lane 7*, vector only cell extract (negative control). Fig. 5B shows a western blot with anti-SLF polyclonal

antibodies (lane designations were the same as for Fig. 5A). Fig. 5C shows a western blot with anti-CBD polyclonal antibodies (lane designations are the same as for Fig. 5A).

Figure 6 shows activity and neutralization of activity of proteins in solution.

5 The proliferative activities of the fusion protein (open squares) and the control protein (open circles) were analyzed (MTT assay) in the absence of an immobilization matrix (Fig. 6A). The activity of 0.6 nM SLF-CBD was neutralized by anti-SLF neutralizing polyclonal antibodies (Fig. 6B). Baseline MTT activity of 0.2 absorbance units was subtracted.

10 Figure 7 shows activity of SLF-CBD as a function of BMCC concentration: the BMCC concentration was varied with SLF/CBD concentration held constant at 130pM (Fig. 7A) or at 1500pM (Fig. 7B). The dotted line represents the activity of SLF-CBD in the absence of cellulose.

Figure 8 shows the activity of SLF-CBD as a function of BMCC
15 concentration. The concentration of SLF-CBD added to each well was varied while the BMCC concentration was held constant at the concentrations indicated. Fig. 8A shows SLF with (closed circles) and without (open circles) 1 μ g/ml BMCC; Fig. 8B shows SLF-CBD without (open circles) or with either 1 μ g/ml BMCC (closed triangles) or 14 μ g/ml BMCC (closed squares).

20 Figure 9 shows separation of protein bound to the matrix from protein not bound to the matrix. Various amounts of SLF-CBD were bound to 1 μ g/ml of BMCC. The matrix was then removed from solution by centrifugation and resuspended in fresh medium. The activities of the original medium and the resuspended BMCC were then assayed for activity to determine how much of the
25 protein bound to the matrix. With control SLF, most of the activity was in the supernatant (Fig. 9A). With SLF-CBD, the bulk of the activity was associated with the BMCC (closed squares) as compared to the supernatant (open squares). The results with unseparated SLF-CBD are shown in the inset (closed squares) (Fig. 9B). When the cellulose affinity tag of SLF/CBD was cleaved from the SLF by Factor Xa
30 the activity was no longer associated with the BMCC (closed squares), but was instead associated with the supernatant (open squares) (Fig. 9C).

Figure 10 shows the activity of SLF-CBD bound to regenerated cellulose. SLF-CBD was bound to a cellulose-coated microtiter plate and the supernatant was removed from each well and replaced with medium. Cells were then added to the plate and the proliferation activity remaining bound in each well was determined (closed triangles) (Fig. 10A). SLF-CBD was bound to cellulose-coated microtiter wells and the wells were used for three consecutive rounds of cell culture (Fig. 10B). MTT assays of cultures following the third use indicate no loss in proliferative activity (closed triangles) as compared to the third use of a non-cellulose surface (open diamonds) (Fig. 10B); a control plate was treated identically except that cells were not added until the third cycle (open triangles).

Figure 11 shows stimulation of murine bone marrow colony formation in response to SCF-CBD. Figs. 11A through 11D show the number of colonies formed by primary murine bone marrow cells when maintained for five days in liquid cultures containing SCF (Figs. 11A and 11B) or SCF-CBD (Figs. 11C and 11D) in the presence of cellulose (b and d) or in the absence of cellulose (a and c). A parallel series of cultures included in addition, IL-3 (filled bars) A) Number of progenitors recovered per 10^4 cells seeded in the liquid cultures. Figures 11C-F are photographs of plates showing larger colony sizes formed by progenitors recovered from liquid culture stimulated by SCF-CBD with cellulose. Scale bar represents 1 mm.

Figure 12 shows anti-phosphotyrosine CLSM of cells cultured with CBD-SCF bound to a *V. ventricosa* cellulose surface. Antibody against phosphotyrosine bound to permeabilized cells (a) and co-localized in patches with anti-receptor antibody (b). A cross-section (Z-section) perpendicular to the cellulose surface passes through the cell at the plane indicated by the arrows in (a) and (b). Activation was primarily localized to the cellulose surface (c) and co-localized with the anti-receptor antibody (d). The scale bar in (c) represents 10 μm ; all panes have the same magnification.

Figure 13 shows CLSM imaging of MO7e cells stimulated with soluble rhSCF. Factor starved cells were stimulated with a range of SCF doses. After 20 minutes, cells were recovered, fixed and then stained with mouse anti-human c-kit (green) and phalloidin (red). Each row shows a series of optical sections ($\delta z \sim 1.0 \mu\text{m}$) through a single, representative cell. Phalloidin-Texas Red binding to the actin cytoskeleton clearly shows the actin envelope of the cell membrane and the

cytoplasmic compartment of the cell volume. Yellow patches within the cytoplasm are the result of actin (red) and c-kit (green) colocalization. The scale bar in the bottom left corner represents 15 μm .

Figure 14 shows live-cell CLSM of B6SutA cells stimulated with FITC-CBD-SCF. Soluble FITC-tagged CBD-SCF was added to cultures and 3-D image sets were collected over time. To minimize the photodamage to cells caused by free radical release from excited fluorophores, confocal imaging was limited to 5 sections at 3 micron spacing. An image stack was collected every 20 seconds for approximately 10 minutes. Presented images are the projections of the middle 3 layers of each image stack. In this figure is demonstrated the time course of receptor patching in response to addition of soluble FITC-tagged SCF to the culture medium (~1.5 minutes between frame). In the first panel (t_0) the slight autofluorescence of the cells is visible. Following addition of labeled CBD-SCF, image brightness increases immediately in all areas of the image field not occupied by cells. A fluorescent halo develops around each cell within seconds of the addition of labeled SCF, followed by the rapid formation of fluorescent aggregates on the cell membrane. Internalized aggregates were discernible within the cell volume within 3 minutes of SCF addition.

Figures 15A through 15C show immunofluorescent laser scanning confocal microscopy of cells grown with BMCC surface presented SCF-CBD. Images were prepared as projections from image stacks prepared with a BioRad MRC600 confocal microscope. Cells were stimulated for 20 minutes with soluble (Fig. 15A) or BMCC bound CBDSCF (Fig. 15B). The fibrous morphology of the BMCC cellulose is evident in Fig. 15B. The localization of the c-kit receptor on this cell is shown in Fig. 15C. Again, the antibody labeling is consistent with the fibrous nature of the BMCC cellulose. Colocalization of receptor and ligand is demonstrated in Fig. 15D. The aquamarine stripes on the cell surface are a result of the colocalization of the SCF (green) and the c-kit receptor (blue). The cell cytoskeleton was counterstained with phalloidin-Texas Red to delineate the cell envelope (red).

Figures 16A and 16B show anti-CBD_{Cex} immunofluorescent CLSM of cells cultured on Valonia surfaces with bound SCF-CBD. B6SutA cells were grown overnight on a Valonia cellulose sheet to which SCF-CBD was bound. After fixing

with 4% paraformaldehyde, cells were labeled with appropriate primary antibody and then stained with fluorescinated secondary antibody or Streptavidin. Imaging was performed at the cellulose-cell interface with a depth of focus of about 1 μm . Antibodies raised against the CBD bound to SCF-CBD on the cellulose surface
5 (Fig. 16A). Labeling intensity increased at the cell and colocalized with antibody against murine c-kit (Fig. 16B).

Figure 17 shows BMCC fibers presenting CBDSCF are internalized by B6SutA cells. B6SutA cells were incubated with BMCC presented CBDSCF for 20 minutes, fixed, and then stained with rabbit anti-SCF antibody (green),
10 biotinylated rat anti-c-kit (blue) and phalloidin (red). The figure shows a rotation series of a volume rendered confocal image stack. Colocalization in this image is represented as the color addition of labels (*e.g.*, aquamarine is green/blue and white is green/blue/red). The arrow points to a fiber near the center of the cell volume.

Figures 18A through 18C show anti-phosphotyrosine immunofluorescent
15 CLSM of cells cultured on Valonia surfaces with bound SCF-CBD. Antibody against phosphotyrosine bound to permeabilized cells (Fig. 18A) and colocalized in patches with anti-receptor antibody (Fig. 18B). A cross section (Z-section) perpendicular to the cellulose surface passing through the cell at the plane indicated by the arrows in (Fig. 18A) and (Fig. 18B). Activation was primarily localized to
20 the cellulose surface (Fig. 18C) and colocalized with the anti-receptor antibody (Fig. 18D). Scale bar in (Fig. 18C) represents 10 μm ; all panels have the same magnification.

Figures 19A and 19B show *V. ventricosa* cell wall sheet labeled with CBD_{Cex}-FITC. Figure 19A shows images of the mounted cellulose sheet stained
25 with the fluorescein labeled binding domain from the exoglucanase Ccs (CBD_{Cex}-FITC). The *ca* 0.5 μm fibers of packed microfibrils are evident. These fibers are stacked into lamella oriented at right angles. Several orthogonal layers make up each sheet. Figure 19B shows a cross section of the surface at the axis indicated in the figure. The sheet is approximately 1.0 μm thick. The image was collected with
30 a 60 X (N.A. 1.4) lens and the scale bar represents 5 μm . the images are the average of three successive scans at a zoom of 2. The confocal aperture on the BioRad 600 was set to 3.

Figure 20 shows a typical isotherm prepared with FITC labeled CBD_{Cex}. Binding parameters derived from a two site model for binding are in good agreement with our earlier studies using unlabeled CBD and bacterial microcrystalline cellulose. About 85% of the binding has a high apparent affinity of about 50 μM^{-1} . The remaining 15% of the sites have a lower affinity of about 1.0 μM^{-1} . Valonia cellulose binds about 6.2 $\mu\text{mole CBD}_{\text{Cex}}$ per gram of cellulose. The inset shows a semilog plot of the isotherm data and the fitted model. Points are means of triplicate binding reactions.

Figures 21A through 21C show an image sequence collected during FRAP analysis. Prior to bleaching an image is collected of the region to be bleached (Zoom = 2) (Figure 21A). This image is analyzed to obtain the initial fluorescence intensities. The CLSM is then electronically zoomed (Zoom = 8) so that only a small region of the surface is illuminated during laser scanning. One scan is performed at this high zoom to produce a large, bleached reference region (Figure 21B). The CLSM zoom is then returned to 2 and six bleached spots are rapidly produced with six successive 100 msec. laser exposures (Figure 21B). Fluorescence intensity is monitored for several minutes until recovery is greater than 95% complete (see Figure 21C). Note the lack of recovery of the central area of the bleached reference region.

Figures 22A through 22C show photobleaching analysis of CBD-FITC on valonia. Time profiles of the fluorescence signal for the bleach spot center (x), the unbleached region of the cellulose surface (o) and the center of the bleached reference region are shown in Figure 22A. Less than 5% background bleaching occurred during monitoring. Figure 22B presents a typical recovery profile used for the estimation of the diffusion coefficient and the mobile fraction of CBD_{Cex} at a surface coverage density of ca. 60%. Fluorescence intensity is normalized by the prebleach fluorescence signal. Under these conditions, the diffusion rate for CBD_{Cex} on crystalline cellulose is $6.0 \pm .5 (10^{-11})\text{cm}^2/\text{sec}$. The mobile fraction of CBD_{Cex} is $70\% \pm 5\%$.

Figures 23A and 23B show surface diffusion rate as a function of bound CBD concentration. Figure 23A presents the measured diffusion coefficient as a function of the fraction of maximal surface coverage $\Gamma/\Gamma_{\text{max}}$. Points are means of 12

individual spot FRAP analysis, error bars show ± 1 standard error from the mean. Diffusion rates were found to increase with Γ/Γ_{\max} . In Figure 23B is presented the measured diffusion coefficient as a function of the fraction of maximal surface coverage Γ/Γ_{\max} . Points are means of 12 individual spot FRAP analysis, error bars show ± 1 standard error from the mean. At low surface coverages the mobile fraction is approximately 60%. As the surface coverage density increases the mobile fraction increases to a maximum of about 80%.

Figure 24 shows the binding of proteins and their CBD-fusions to different polymers. 100 μ l triplicates of a 100 μ g/ml protein solution containing either GFP, CBDclos-GFP, Steptavidin and CBDclos-Streptavidin were incubated with EHEC coated polystyrene, polystyrene and polypropylene microtiter plates at RT for 2 hours. After successive washing with PBS, the amount of protein bound to the different plates was determined by the Micro-BCA assay at 562 nm.

Figure 25 shows the binding of Steptavidin and CBD-Streptavidin fusion protein to EHEC coated and uncoated polystyrene plates. Polystyrene microtiter plates were coated with EHEC-230 and EHEC-481. The EHEC coated and uncoated polystyrene plates were incubated with Streptavidin and CBDclos-Streptavidin at RT for 2 hours, and the unbound protein was washed out with PBS. Biotinylated HRP was used to detect the amount of Streptavidin bound to the plates. The signal intensity was measured by ELISA at 405 nm.

Figure 26 shows bound CBD on cellulose (μ mole CBD/g cellulose) plotted against free CBD (μ M).

Figure 27 shows that cellulose enhances the activity of SCF-CBD. A dose response assay of cell proliferation was carried out for SCF-CBD (squares) and SCF (circles) in B6SutA cell cultures with (filled symbols) or without (open symbols) cellulose. SCF-CBD was more potent when localized on cellulose.

Figure 28 shows the viability of B6SutA cells grown in Iscove's modified Dulbecco's medium containing 10% FCS and 5% HemoStim™ M2100 after 48 hours when exposed to various levels of G418 ranging from a lowest level 0.01 mg/ml. Viability as a percentage (%) of cells originally exposed to the G418 is plotted against the G418 level.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention provides methods and compositions for *ex vivo* cultivation of growth-factor dependent cells, particularly cells of the hematopoietic system such as stem cells and T-lymphocytes. By a growth factor is intended a factor, generally a polypeptide, which stimulates or otherwise modulates proliferation or differentiation of a target cell of interest having a cell surface receptor to which the growth factor binds. Included is the large class of soluble growth factors that are derived from membrane-anchored precursors that include an extracellular region containing the growth-factor domain, a hydrophobic transmembrane domain, and a small cytoplasmic domain. The soluble forms of these growth factors are produced *in vivo* by proteolytic cleavage of the precursor's extracellular region. A target cell of interest is any cell in which proliferation and/or differentiation is modified by the growth factor, including stimulation and blocking the proliferation. The compositions also find use for isolating growth-factor receptors and/or the cells containing them, and in normal healing. The compositions include chimeric molecules comprising a growth factor and an amino acid sequence that is capable of binding to a polysaccharide substrate, generally with high affinity. The polysaccharide substrates include β -glycans, in particular β -1,4-glycans, including cellulose and chitin. In use, the chimeric molecule is immobilized on the polysaccharide substrate, which can be either soluble or insoluble, and cells dependent upon the growth factor are grown in contact with the chimeric molecule. If increased proliferation of a target cell is the desired result, the chimeric molecule is presented to the cell in a form which effects transient activation of the growth-factor receptor. This may be accomplished by binding the cytokine to a particulate polysaccharide matrix or other polysaccharide matrix, which permits internalization of the cytokine. Internalization of the receptor along with the growth factor will result, for example, by use of a soluble polysaccharide substrate or a form of cellulose, which can be internalized, such as microcrystalline cellulose. Internalization of the receptor bound to the cytokine-CBD/polysaccharide complex results in transient proliferation of the cell. Such transient stimulation of cell proliferation is more effective than stimulation with the chimeric cytokine-CBD fusion protein in the absence of any polysaccharide matrix to which the chimera binds.

If cell differentiation and/or cell maintenance, rather than cell proliferation or expansion is desired, the cytokine-CBD fusion protein is presented in a form which prolongs activation of the receptor. For example, the chimeric protein bound to an insoluble and non-internalizable polysaccharide substrate or any other non-internalizable surface to which the chimeric molecule binds.

The invention also provides methods for obtaining a population of cells enriched in growth-factor dependent cells. These methods are useful for obtaining purified preparations of, for example, stem cells (including pluripotent stem cells) and other relatively rare cell types. The methods involve contacting a plurality of cells with a growth-factor conjugate and then removing any cells that lack the cell surface receptor. The cells having receptors for the growth factor of interest bind specifically to the growth-factor conjugates, while other non-growth-factor dependent cells do not bind to the growth-factor conjugates in significant amounts. The bound cells can be used while still bound to the growth-factor conjugates; alternatively, the cells can be eluted from the growth-factor conjugates to obtain a purified preparation of growth-factor dependent cells. Amplification of these cells then can be accomplished by the specific growth stimulation provided by a growth-factor conjugate which provides for transient activation of the growth-factor receptor so as to preferentially increase proliferation of responsive cells.

The growth-factor conjugates described herein can also be used for enhancing healing of a wound. This is accomplished by contacting the wound with the growth-factor conjugate that, in this instance, comprises an agent that either stimulates local proliferation of cells involved in wound healing, and/or is chemotactic for such cells. For example SCF-CBD immobilized on an Avicel matrix has been observed to exert a chemotactic attraction upon B6SUtA cells. The growth-factor conjugate is optionally bound to a wound covering, preferably a covering that comprises an insoluble cellulose matrix such as a sponge or a cotton bandage, via a cleavage site, such as a protease cleavage site, so that the growth factor can be internalized by the cell. Other possible wound coverings which comprise a matrix to which the growth-factor-CBD chimera may be bound for enhancement of wound healing include PASA mixed with gelatin, chitin, chitosan, mucopolysaccharides, starch gels and ethyl hydroxy ethylcellulose (EHEC).

The use of immobilized growth factors provides several advantages over non-immobilized growth factors and currently used methods for immobilizing growth factors and other biological molecules that modulate cell growth, differentiation, and/or proliferation. The initial event in the stimulation of many cell types is the clustering of receptors following their interaction with cytokines or growth factors. Presentation of the cytokines on stromal cells is particularly effective for triggering these responses, presumably because lateral mobility of the cytokines on the surfaces of the presenting cells allows clustering of receptors on the target cells. However, stromal cells produce a variety of molecules, any or all of which may affect the target cells. The method described here allows facile presentation of a single cytokine in the absence of any confounding effects of unknown factors. The advantage of the method is that the mobility of the CBD fusion protein on the cellulose surface, like the lateral movement of cytokines on stromal cells, allows receptor patching on the target cells. Furthermore, adsorption of the cytokine-CBD fusion to cellulose ensures its presentation in a uniform orientation. Previous efforts to immobilize cytokines and growth factors on culture surfaces involve covalent attachments (Cuatrecasas, (1969) *Proc. Natl. Acad. Sci. USA* 63:450-457; Ito *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93:3598-3601; Kuhl and Griffith-Cima, (1996) *Nature Medicine* 2:1022-1027; Horwitz *et al.*, (1993) *Molec. Immun.* 30:1041-1048) which precludes lateral diffusion. When IL-2 was attached to a culture surface covalently, division of CTLL cells was effectively abolished but could be reinitiated by addition of soluble IL-2 (Horwitz, *supra*).

Immobilized growth factors prepared as in the subject invention thus can provide a specific stimulus for cell proliferation when presented in a form which can be internalized by the target cell. Immobilized growth factors prepared as in the subject invention also may be employed to provide a specific stimulus for differentiation when presented in a form which would not be expected to be internalized by the target cell. One example of a surface which is not expected to be internalizable is the SCF-CBD/Valonia surface to which cell receptors have been observed to remain attached without their cells. Furthermore, when immobilized growth factors are used bound to a substrate that is not consumed by cells, the growth factor remains available to stimulate differentiation of additional cells. This is

particularly useful in perfusion cultures in which growth medium is continuously added and removed to allow long term cell maintenance. Moreover, an immobilized growth factor is often more active than a soluble version of the same growth factor.

One of the major advantages of the CBD-cytokine system is the ability to specifically adsorb active growth factor to cellulose materials which are available in a wide variety of geometries and conformations. These can be exploited in order to prepare specific patterns and distributions of growth factors for culturing cells into specific geometries.

For example, CBD-EGF (or other neural active growth factor such as CBD-netrin, CBD-NT-3, CBD-NGF) is adsorbed to cellulose fibrils. This fibril is then laid out on a cell culture surface using standard micromanipulation techniques. The fibrils are positioned into the pattern in which the neural cells are desired to grow. For example, the fibers could be laid out so that axons are stimulated to grow out linearly from the cell body. This construct would be useful when it is desired to close some gap in a nervous systems connection (perhaps arising from severing of a nerve fiber).

Another application uses a cellulose fiber with appropriate CBD-growth factor surrounded by a cell culture compatible polymer matrix. For example, a tube is filled with extracellular matrix components (polymer gel) and the CBD-growth factor fiber is threaded through the tube. The tube is now ready for transplant into the site where a nerve has been severed. Because the growth factor on the cellulose fiber presents the spatial cues for growing through the tube, the inserted tube acts as a bridge to direct axon outgrowth (to bridge the nerve break) and acts as a support to stabilize the position of the nerve with respect to target nerves for synapse formation.

The system described here also has advantages in terms of design flexibility. A CBD can be attached to the N or C terminus of the cytokine (Assouline *et al.*, (1993) *Protein Eng.* 6:787-792; Greenwood *et al.*, (1994) *Biotech. Bioeng.* 44:1295-1305) or be located between two molecules (Tomme *et al.*, (1993) *Protein Eng.* 7:117-123), allowing either presentation of a single factor in either orientation relative to the surface or of multiple factors simultaneously. Furthermore, our results indicate that the correct density of cytokines on the cellulose surface is crucial for maximal cell stimulation. For MO7e cells, this density was found to be $\sim 10^{10}$ SCF-CBD molecules cm^{-2} , roughly equal to the receptor density on the cell surface (Turner *et al.*,

(1995) *Blood* 85:2052-2058). Using the cellulose/CBD-fusion system, both the area of the presenting surface and the density of factor on the surface can be varied.

Additional advantages provided by the subject invention include the following. Oligosaccharide polymers including carbohydrate polymers such as
5 cellulose and other β -glycans, such as those obtained from oat and barley, are plentiful and inexpensive. Furthermore, a variety of proteins bind specifically to carbohydrate polymers and other oligosaccharides and can be used as the source of polysaccharide binding peptides (PBPs) for the subject invention. As an example, fusion proteins can be prepared which include the carbohydrate polymer-binding portion of a protein
10 which binds to a carbohydrate as a means for immobilizing the fusion protein. Thus, use of the PBP provides a generic means for immobilization of any growth factor or related moiety by attaching it to a PBP which can bind to a polysaccharide. The selective binding of the PBP to the oligosaccharide polymer makes it especially suitable for the purification and/or immobilization of a wide variety of compounds.

15 Juxtacrine stimulation of adjacent cells by membrane-localized factors is initiated by contact and binding of complementary receptors presented on the surfaces of neighboring cells. Activation of the receptor's intracellular tyrosine kinase, also requires dimerization of adjacent factor-receptor complexes. This dimerization event suggests that both the factor and the complementary receptor are free to diffuse within
20 the cell membrane. Engineered biomaterials that localize growth factors (or other stimulants) in a manner which allows for surface diffusion of the ligand therefore offer advantages over solid-phase systems, currently targeted for wound-healing or tissue-regeneration applications, in which the factor is covalently bound to the surface.

25 Dynamic-stimulant localization also offers the advantage that it enhances development of hematopoietic cell culturing systems for applications in bone-marrow transplantation, gene therapy and the production of blood products. Current *ex vivo* expansion systems for hematopoietic cells are primarily based on stromal feeder cell layer cultures. Dynamic localization reduces the demand for growth factors in these
30 systems by presenting the factors in a tunable surface-concentrated form which is not susceptible to endocytosis, but allows for stimulation of progenitor cells through receptor dimerization and patching.

The invention provides novel polypeptide compositions that can include those having the following formula:



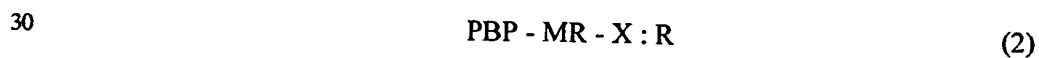
wherein:

5 PBP is characterized as a consecutive sequence of amino acids from the substrate binding region of a polysaccharidase or other peptide or protein which binds to a polysaccharide substrate to provide for binding to a substrate of the polysaccharidase and, optionally, essentially lacking in polysaccharidase activity is at least as large as the minimum number of amino acids in a sequence required to bind a
10 polysaccharide;

MR is the middle region, and can be a bond; short linking group of from 2 to 30 carbon atoms, or have from about 2 to about 20 amino acids. The region can include an amino acid sequence providing for specific cleavage of the fusion protein that comprises the growth-factor conjugate, usually a sequence corresponding to that
15 recognized by a proteolytic enzyme of high specificity such as an IgA1 protease or Factor Xa; and

X can be any growth factor or other moiety that stimulates or inhibits cell proliferation or activation through binding to a cell surface receptor. X is characterized as having up to the entire sequence of a polypeptide of interest; X can
20 have only that part of the polypeptide that is required to exert the desired effect on cell proliferation. X can be, for example, a cytokine, lymphokine, or other growth factor. For example, suitable growth factors include a steel factor, an interleukin-2, an interleukin-3, an interleukin-6, an interleukin-11, a mast cell growth factor, a granulocyte colony stimulating factor, a granulocyte-macrophage colony stimulating
25 factor, a fibroblast growth factor, a platelet-derived growth factor, or an epidermal growth factor. X indicates only the moiety, not the stoichiometry of the moiety, which can be variable.

PBP-MR-X can be bound to an isolated receptor R for X, or to cells containing R, wherein the composition has the formula:



wherein R can be any moiety that is present on a cell surface and which, when contacted by a corresponding ligand (X), specifically binds R. R and X are the first

and second members, respectively, of a specific binding pair. Generally, the interaction between X and R is noncovalent, such as electrostatic, hydrogen bonding, polar/nonpolar interactions, and the like. R can be, for example, a growth-factor receptor, a T cell receptor, an immunoglobulin, or an MHC polypeptide. Specific
5 binding of the first member to the second member of a specific binding pair generally is of high affinity, namely on the order of 10^{-8} to 10^{-11} M when R is a growth-factor receptor.

A characteristic of the specific binding pairs used in the subject invention is that the interaction of the second member with the first member exerts an effect either
10 directly or indirectly on the cell upon which the first member is present. For example, interaction of a binding pair wherein the second member is a growth factor and its corresponding receptor is the first member can induce a cell to proliferate or to differentiate. Examples of growth factors of interest are steel factor, an insulin-like growth factor, epidermal growth factor, Fibroblast growth factor, nerve growth factor,
15 FLT 3 ligand, a nerve cell growth factor such as brain derived neurotrophic factor, an osteogenic growth factor, erythropoietin, GM-CSF, G-CSF, M-CSF; interleukins (including IL-2, IL-3, IL-6 and others), an angiogenesis factor, a blood forming factor, and the like. Interaction between the members of other specific binding pairs can cause activation or inactivation of cell division, or can induce a cell to differentiate.
20 For example, an appropriate MHC polypeptide can interact with a T cell receptor to induce clonal expansion of a T cell population. Other binding pairs can serve as chemotactic signals that direct cell migration. Several growth factors have been shown to be chemotactic for their respective target cell, including platelet derived growth factor (PDGF) for neutrophyls and fibroblasts, IL-2 for T-lymphocytes,
25 (particularly activated $CD4^{+}$ T-lymphocytes), insulin for T-cells (both $CD4^{+}$ and $CD8^{+}$) as well as unactivated T-cells. This characteristic has been exploited in wound healing. Other examples of chemotactic moieties include cell adhesion receptors such as integrins, LAM-1, ICAM-1, LFA-3, H-CAM, ELAM-1, and their corresponding receptors or ligands.

30 Either or both members of a specific binding pair can be comprised of a polypeptide and/or a carbohydrate. Carbohydrates that are useful in the subject invention include those, such as sialyl-Lewis^x (SLe^x) and other carbohydrate moieties

involved in cell adhesion and signaling, that specifically bind ligands. For example, a conjugate that comprises a ligand for SLe^x (such as ELAM-1) linked to a substrate binding region derived from a polysaccharidase is useful for purifying cells that display SLe^x on their surfaces, and also for purifying SLe^x-containing
5 oligosaccharides and proteins to which such oligosaccharides are linked.

Alternatively, a carbohydrate ligand such as SLe^x can be linked to a substrate binding region derived from a polysaccharidase; such conjugates are useful for attracting cells that display on their surfaces a ligand that binds SLe^x. Immobilizing these conjugates on a polysaccharide support such as a wound covering thus provides a method of
10 directing cells involved in, for example, fighting infection, to a wound.

For some applications, a conjugate that is bifunctional is used. The bifunctional conjugate includes more than one growth factor attached to the PBP, usually two growth factors. These conjugates include a second second member of a specific binding pair in addition to the first second member of a specific binding pair.
15 The second second member generally is a different growth factor from the first second member. Both the first and second second members are attached to the PBP. An example of such a bifunctional growth-factor conjugate is a PBP to which is bound steel factor and one or more growth factors, preferably a growth factor with which steel factor acts synergistically, such as IL-3, IL-11, GM-CSF, and/or EPO.

20 The PBPs include amino acid sequences that are derived from or obtainable from a substrate binding region (SBD; also referred to herein as a polysaccharide binding domain (PBD)) of a polysaccharidase. The polysaccharide binding peptide can include any amino acid sequence which binds to an oligosaccharide polymer, for example, the PBP can be derived from a SBD of a polysaccharidase, a binding domain
25 of a polysaccharide binding protein or a protein designed and engineered to be capable of binding to a polysaccharide. The PBP can be naturally occurring or synthetic. Suitable polysaccharidases from which a PBP or SBD may be obtained include β -1-4-glucanases. In a preferred embodiment, a PBP or SBD from a cellulase or chitinase is used. Typically, the amino acid sequence is essentially lacking in the hydrolytic
30 activity of the polysaccharidase, but retains the substrate binding activity. The amino acid sequence preferably has less than about 10% of the hydrolytic activity of the

native polysaccharidase; more preferably less than about 5%, and most preferably less than about 1% of the hydrolytic activity of the native polysaccharidase.

The PBP can be obtained from a variety of sources, including enzymes which bind to oligosaccharides which find use in the subject invention. In Table 5 below are listed those binding domains which bind to one or more soluble/insoluble polysaccharides including binding domains with affinity for soluble glucans (α , β , and/or mixed linkages). The N1 cellulose-binding domain from endoglucanase CenC of *C. fimi* binds soluble cellosaccharides; endoglucanase CenC of *C. fimi* is one of a small set of proteins which are known to bind any soluble polysaccharides. Also, listed in Tables 1 to 4 are examples of proteins containing putative β -1,3-glucan-binding domains (Table 1); proteins containing Streptococcal glucan-binding repeats (Cpl superfamily) (Table 2); enzymes with chitin-binding domains (Table 3), and starch-binding domains (Table 4). Scaffolding proteins which include a cellulose binding domain protein such as that produced by *Clostridium cellulovorans* (Shoseyov *et al.*, PCT/US94/04132) also can be used for preparing a PBP. Several fungi, including *Trichoderma* species and others, also produce polysaccharidases from which PBP can be isolated.

Table 1

Overview of proteins containing putative β -1,3 glucan-binding domains

Source (strain)	Protein	accession N°	Ref ¹
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Type I

<i>B. circulans</i> (WL-12)	GLCA1	P23903/M34503/JQ0420	1
<i>B. circulans</i> (IAM 1165)	BglH	JN0772/D17519/S67033	2

¹. References

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- 2) Yamamoto *et al.* (1993) *Biosci. Biotechnol. Biochem.* 57, 1518-1525
- 3) Harpin *et al.* (1994) *EMBL Data Library*
- 4) Shen *et al.* (1991) *J. Biol. Chem.* 266, 1058-1063
- 5a) Shimoi *et al.* (1992) *J. Biol. Chem.* 267, 25189-25195
- 5b) Shimoi *et al.* (1992) *J. Biochem* 110, 608-613
- 6) Horn *et al.* (1989) Patent A12892
- 7) Shareck *et al.* (1991) *Gene* 107, 75-82
- 8) Seki *et al.* (1994) *J. Biol. Chem.* 269, 1370-1374
- 9) Watanabe *et al.* (1993) *EMBL Data Library*

Type II

5	<i>Actinomadura</i> sp. (FC7)	XynII	U08894	3
	<i>Arthrobacter</i> sp. (YCWD3)	GLCI	D23668	9
	<i>O. xanthineolytica</i> GLC	P22222/M60826/A39094	4	
	<i>R. faecitabidus</i> (YLM-50)	RP I		
10		Q05308/A45053/D10753	5a,b	
	<i>R. communis</i>	Ricin A12892	6	
	<i>S. lividans</i> (1326)	XlnA P26514/M64551/JS07986	7	
	<i>T. tridentatus</i>	FactorGa D16622	8	

15 *B.* : *Bacillus*, *O.* : *Oerskovia*, *R. faecitabidus* : *Rarobacter faecitabidus*,
R. communis: *Ricinus communis*, *S.* : *Streptomyces*, *T.* : *Tachypleus*
(Horseshoe Crab)

Table 2

20 Overview of proteins containing Streptococcal glucan-binding repeats
(Cpl superfamily)

25	Source	Ref. ³	Protein	Accession N°	
	<i>S. downei</i> (<i>sobrinus</i>) (0MZ176)		GTF-I	D13858	
		1			
30	<i>S. downei</i> (<i>sobrinus</i>) (MFe28)		GTF-I	P11001/M17391	
		2			
	<i>S. downei</i> (<i>sobrinus</i>) (MFe28)		GTF-S		
		P29336/M30943/A41483	3		
35	<i>S. downei</i> (<i>sobrinus</i>) (6715)		GTF-I		
		P27470/D90216/A38175	4		
	<i>S. downei</i> (<i>sobrinus</i>)		DEI	L34406	5
	<i>S. mutants</i> (Ingbritt)		GBP	M30945/A37184	6
	<i>S. mutants</i> (GS-5)		GTF-B	A33128	
		7			
40	<i>S. mutants</i> (GS-5)		GTF-B		
		P08987/M17361/B33135	8		
	<i>S. mutants</i>		GTF-B ^{3'-ORF}	P05427/C33135	
		8			
	<i>S. mutants</i> (GS-5)		GTF-C		
45		P13470/M17361/M22054	9		
	<i>S. mutants</i> (GS-5)		GTF-C	not available	
		10			
	<i>S. mutants</i> (GS-5)		GTF-D	M29296/A45866	

23

11

	<i>S. salivarius</i>	GTF-J	
	A44811/S22726/S28809	12	
5		Z11873/M64111	
	<i>S. salivarius</i>	GTF-K	
	S22737/S22727/Z11872	13	
	<i>S. salivarius</i> (ATCC25975)	GTF-L	L35495
		14	
10	<i>S. salivarius</i> (ATCC25975)	GTF-M	L35928
		14	
	<i>S. pneumoniae</i> R6	LytA	
	P06653/A25634/M13812	15	
15	<i>S. pneumoniae</i>	PspA	A41971/M74122
		16	
	Phage HB-3	HBL	P32762/M34652
	17		
20	Phage Cp-1	CPL-1	
	P15057/J03586/A31086	18	
	Phage Cp-9	CPL-9	
	P19386/M34780/JQ0438	19	
	Phage EJ-1	EJL	A42936
25	20		
	<i>C. difficile</i> (VPI 10463)	ToxA	
	P16154/A37052/M30307	21	
		X51797/S08638	
30	<i>C. difficile</i> (BARTS W1)	ToxA	
	A60991/X17194	22	
	<i>C. difficile</i> (VPI 10463)	ToxB	
	P18177/X53138/X60984	23,24	
		S10317	
35	<i>C. difficile</i> (1470)	ToxB	S44271/Z23277
	25,26		
	<i>C. novyi</i>	a-toxin	S44272/Z23280
	27		
40	<i>C. novyi</i>	a-toxin	Z48636
	28		
	<i>C. acetobutylicum</i> (NCIB8052)	CspA	S49255/Z37723
	29		
45	<i>C. acetobutylicum</i> (NCIB8052)	CspB	Z50008
	30		
	<i>C. acetobutylicum</i> (NCIB8052)	CspC	Z50033
	30		

C. acetobutylicum (NCIB8052) CspD Z50009
30

5 ³References

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- 2) Ferreti *et al.* (1987) *J. Bacteriol.* 169, 4271-4278
- 3) Gilmore *et al.* (1990) *J. Infect. Immun.* 58, 2452-2458
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- 6) Banas *et al.* (1990) *J. Infect. Immun.* 58, 667-673
- 7) Shiroza *et al.* (1990) *Protein Sequence Database*
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- 9) Ueda *et al.* (1988) *Gene* 69, 101-109
- 15 10) Russel (1990) *Arch. Oral. Biol.* 35, 53-58
- 11) Honda *et al.* (1990) *J. Gen. Microbiol.* 136, 2099-2105
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- 25 20) Diaz *et al.* (1992) *J. Bacteriol.* 174, 5516-5525
- 21) Dove *et al.* (1990) *J. Infect. Immun.* 58, 480-488
- 22) Wren *et al.* (1990) *FEMS Microbiol. Lett.* 70, 1-6
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- 30 25) Sartinger *et al.* (1993) *EMBL Data Library*
- 26) von Eichel-Streiber *et al.* (1995) *Mol. Microbiol.* In Press
- 27) Hofmann *et al.* (1993) *EMBL Data Library*
- 28) Hofmann *et al.* (1995) *Mol. Gen. Genet.* In Press
- 29) Sanchez *et al.* (1994) *EMBL Data Library*
- 35 30) Sanchez *et al.* (1995) *EMBL Data Library*

New PBPs with interesting binding characteristics and specificities can be identified and screened for in a variety of ways including spectroscopic (titration) methods such as: NMR spectroscopy (Zhu *et al. Biochemistry* (1995) 34:, Gehring *et al. Biochemistry* (1991) 30:5524-5531), UV difference spectroscopy (Belshaw *et al. Eur. J. Biochem.* (1993) 211:717-724), fluorescence (titration) spectroscopy (Miller *et al. J. Biol. Chem.* (1983) 258:13665-13672), UV or fluorescence stopped flow analysis (De Boeck *et al. Eur. J. Biochem.* (1985) 149:141-415), affinity methods such as affinity electrophoresis (Mimura *et al. J. chromatography* (1992) 597:345-

350) or affinity chromatography on immobilized mono or oligosaccharides, precipitation or agglutination analysis including turbidimetric or nephelometric analysis (Knibbs *et al. J. Biol. Chem.* (1993) 14940-14947), competitive inhibition assays (with or without quantitative IC50 determination) and various physical or
5 physico-chemical methods including differential scanning or isothermal titration calorimetry (Sigurskjold *et al. J. Biol. Chem.* (1992) 267:8371-8376; Sigurskjold *et al. Eur. J. Biol.* (1994) 225:133-141) or comparative protein stability assays (melts) in the absence or presence of oligosaccharides using thermal CD or fluorescence spectroscopy.

10 Generally, for use in constructing immobilized growth factors, the K_a for binding of the PBP to oligosaccharide is at least in the range of weak antibody-antigen interactions, *i.e.*, 10^3 , preferably 10^4 , most preferably 10^6 . If the binding of the PBP to the oligosaccharide is exothermic or endothermic, then binding will increase or decrease, respectively, at lower temperatures,
15 providing a means for temperature modulation of the immobilization step.

Table 3
Overview of Enzymes with Chitin-Binding Domains

Source (strain)	Enzyme	Accession N°	Ref. ⁴
Bacterial enzymes			
<u>Type I</u>			
10 <i>Aeromonas</i> sp. (No10S-24)	Chi	D31818	1
<i>Bacillus circulans</i> (WL-12)	ChiA1	P20533/M57601/A38368	2
<i>Bacillus circulans</i> (WL-12)	ChiD	P27050/D10594	3
<i>Janthinobacterium lividum</i>	Chi69	U07025	4
15 <i>Streptomyces griseus</i>	ProteaseC	A53669	5
<u>Type II</u>			
20 <i>Aeromonas cavia</i> (K1)	Chi	U09139	6
<i>Alteromonas</i> sp (0-7)	Chi85	A40633/P32823/D13762	7
<i>Autographa californica</i> (C6)	NPH-128 ^a	P41684/L22858	8
<i>Serratia marcescens</i>	ChiA	A25090/X03657/L01455/P07254	9
<u>Type III</u>			
25 <i>Rhizopus oligosporus</i> (IFO8631)	Chi1	P29026/A47022/D10157/S27418	10
<i>Rhizopus oligosporus</i> (IFO8631)	Chi2	P29027/B47022/D10158/S27419	10
<i>Saccharomyces cerevisiae</i>	Chi	S50371/U17243	11
<i>Saccharomyces cerevisiae</i> (DBY939)	Chi1	P29028/M74069	12
30 <i>Saccharomyces cerevisiae</i> (DBY918)	Chi2	P29029/M7407/B41035	12

^aNHP : nuclear polyhedrosis virus endochitinase like sequence; Chi : chitinase

Table 3 - cont'd

Source (strain)	Enzyme	Accession N°	Ref. ⁴
Plant enzymes			
<u>Hevein superfamily</u>			
10 <i>Allium sativum</i>	Chi	M94105	13
<i>Amaranthus caudatus</i>	AMP-1 ^b	P27275/A40240	14, 15
<i>Amaranthus caudatus</i>	AMP-2 ^b	S37381/A40240	14, 15
<i>Arabidopsis thaliana</i> (CV. colombia)	ChiB	P19171/M38240/B45511	16
<i>Arabidopsis thaliana</i>	PHP ^c	U01880	17
15 <i>Brassica napus</i>	Chi	U21848	18
<i>Brassica napus</i>	Chi2	Q09023/M95835	19
<i>Hevea brasiliensis</i>	Hev1 ^d	P02877/M36986/A03770/A38288	20, 21
<i>Hordeum vulgare</i>	Chi33	L34211	22
<i>Lycopersicon esculentum</i>	Chi9	Q05538/Z15140/S37344	23
20 <i>Nicotiana tabacum</i>	CBP20 ^e	S72424	24
<i>Nicotiana tabacum</i>	Chi	A21091	25
<i>Nicotiana tabacum</i> (cv. Havana)	Chi	A29074/M15173/S20981/S19855	26
<i>Nicotiana tabacum</i> (FB7-1)	Chi	JQ0993/S0828	27
<i>Nicotiana tabacum</i> (cv. Samsun)	Chi	A16119	28
25 <i>Nicotiana tabacum</i> (cv. Havana)	Chi	P08252/X16939/S08627	27
<i>Nicotiana tabacum</i> (cv. BY4)	Chi	P24091/X51599/X64519/S13322	26, 27, 29
<i>Nicotiana tabacum</i> (cv. Havana)	Chi	P29059/X64518/S20982	26
<i>Oryza sativum</i> (IR36)	ChiA	L37289	30
<i>Oryza sativum</i>	ChiB	JC2253/S42829/Z29962	31
30 <i>Oryza sativum</i>	Chi	S39979/S40414/X56787	32

33
34
35

X56063
P24626/X54367/S14948
P25765/S15997
D16223

Chi
Chi1
Chi2
Chi3

Oryza sativum (cv. Japonicum)
Oryza sativum (cv. Japonicum)
Oryza sativum
Oryza sativum (cv. Japonicum)

Table 3 - cont'd

Source (strain)	Enzyme	Accession N°	Ref. ⁴
5 <i>Oryza sativum</i>	ChiA	JC2252/S42828	30
<i>Oryza sativum</i>	ChiI	D16221	32
<i>Oryza sativum</i> (IR58)	Chi	U02286	36
<i>Oryza sativum</i>	Chi	X87109	37
<i>Pisum sativum</i> (cv. Birte)	Chi	P36907/X63899	38
<i>Pisum sativum</i> (cv. Alcan)	Chi2	L37876	39
<i>Populus trichocarpa</i>	Chi	SI8750/S18751/X59995/P29032	40
<i>Populus trichocarpa</i> (H11-11)	Chi	U01660	41
<i>Phaseolus vulgaris</i> (cv. Saxa)	Chi	A24215/S43926/Jq0965/P36361	42
<i>Phaseolus vulgaris</i> (cv. Saxa)	Chi	P06215/M13968/M19052/A25898	43,44,45
15 <i>Sambucus nigra</i>	PR-3 ^f	Z46948	46
<i>Secale cereale</i>	Chi	JC2071	47
<i>Solanum tuberosum</i>	ChiB1	U02605	48
<i>Solanum tuberosum</i>	ChiB2	U02606	48
<i>Solanum tuberosum</i>	ChiB3	U02607/S43317	48
20 <i>Solanum tuberosum</i>	ChiB4	U02608	48
<i>Solanum tuberosum</i> (cv. Maris Piper)	WIN-1 ^s	P09761/X13497/S04926	49
<i>Solanum tuberosum</i> (cv. Maris Piper)	WIN-2 ^s	P09762/X13497/S04927	49
<i>Triticum aestivum</i>	Chi	S38670/X76041	50
<i>Triticum aestivum</i>	WGA-1 ^b	P10968/M25536/S09623/S07289	51,52
25 <i>Triticum aestivum</i>	WGA-2 ^b	P02876/M25537/S09624	51,53
<i>Triticum aestivum</i>	WGA-3 ^b	P10969/J02961/S10045/A28401	54
<i>Ulmus americana</i> (NPS3-487)	Chi	L22032	55
<i>Urtica dioica</i>	AGL ⁱ	M87302	56
<i>Vigna unguiculata</i> (cv. Red caloona)	ChiI	X88800	57

^aanti-microbial peptide, ^cpre-hevein like protein, ^dhevein, ³chitin-binding protein, ^fpathogenesis related protein, ^gwound-induced protein, ^hwheat germ agglutinin, ⁱagglutinin (lectin)

³References Chitin-binding domains

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Table 4
Overview of Enzymes Containing Starch-binding Domains

Source (strain)	Enzyme	Accession N°	Ref. ⁵
<i>A. awarori</i> (var. <i>kawachi</i>)	AMYG	P23176/D00427/JT0479	1, 2
<i>A. niger</i> (T21)	AMYG	S73370	3
10 <i>A. niger</i> - <i>A. awamori</i>	AMYG1/G2	P04064/A90986/A29166/X00712/X00548 K02465	4,5,6 7,8,9
<i>A. oryzae</i>	AMYG (GLAA)	P36914/JQ1346/D01035/S75274/D01108	10, 11
<i>A. Shirousamii</i>	AMYG (GLA)	P22832/JQ0607/D10460	12
<i>Bacillus</i> sp. (B1018)	AMY ^a	P17692/M33302/D90112/S09196	13
15 <i>Bacillus</i> sp. (TS-23)	a-AMY	U22045	14
<i>Bacillus</i> sp. (1-1)	CGT	P31746/S26399	15
<i>Bacillus</i> sp. (6.63)	CGT	P31747/X66106/S21532	16
<i>Bacillus</i> sp. (17-1)	CGT	P30921/M28053/A37208	17
<i>Bacillus</i> sp. (38-2)	CGT	P09121/M19880/D00129/S24193	18, 19
20 <i>Bacillus</i> sp. (1011)	CGT	P05618/A26678/M17366	20
<i>Bacillus</i> sp. (DSM5850)	CGT	A18991	21
<i>Bacillus</i> sp. (KC 201)	CGT	D13068	15, 22
<i>B. cereus</i> (SPOII)	b-AMY	A48961/P36924/S54911	23
<i>B. circulans</i> (8)	CGT	P30920/X68326/S23674	24
25 <i>B. circulans</i> (251)	CGT	X78145	25
<i>B. Licheniformis</i>	CGTA	P14014/X15752/S15920	26
<i>B. macerans</i> (IFO 3490)	CGTM (CDG1)	P04830/X5904/S31281	27
<i>B. macerans</i> (IAM 1243)	CGT	M12777	28
<i>B. macerans</i>	CGT (CDG2)	P31835/S26589	29
30 <i>B. ohbenensis</i>	CGT	P27036/D90243	30

31
32

P19531/M36539/S28784
P31797/X59042/S26588/X59043/
X59404/S31284

AMYM^b
CGT

B. stearothermophilus
B. stearothermophilus (NO2)

Table 4 - cont'd

Source (strain)	Enzyme	Accession N°	Ref. ⁵
5 <i>C. rolfsii</i> (AHU 9627)	AMYG2	D4948	33
<i>D. discoideum</i>	ORF	S15693/X51947	34
<i>H. grisea</i> (var. <i>thermoidea</i>)	GLAI	M89475	35
<i>H. resinae</i> (ATCC20495)	GAMP	Q03045/X68143/X67708/S31422/S33908	36-38
10 <i>K. pneumoniae</i> (M5A1)	CGT	P08704/M15264/A29023	39
<i>N. crassa</i> (74-OR23-1A)	GLA-1	P14804/X67291/S13711/S13710/S36364	40, 41
<i>P. saccharophila</i> (IAM1504)	MTA ^c	P22963/X16732/S05667	42
15 <i>Pseudomonas</i> sp. (KO-8940)	AMF-1 ^d	D10769/JS0631/D01143	43
<i>P. stutzeri</i> (MO-19)	AMYP ^c	P13507/M24516/A32803	44
<i>S. griseus</i> (IMRU 3570)	AMY	P30270/X57568/S14063	45
<i>S. limosus</i> (S.albidoflavus)	AML	P09794/ M18244/B28391	46
<i>S. violaceus</i> (S. venezuela)(ATCC15068)	AML	P22998/M25263/JS0101	47
20 <i>Th. curvata</i> (CCM 3352)	TAM ^c	P29750/X59159/JH0638	48
<i>Th. thermosulfurogenes</i> (DSM3896/EM1) ^f	AMYA	P26827/X54654/X54982/S17298/S37706	49
<i>Th. thermosulfurogenes</i> (ATCC 33743)	AMYB	P19584/M22471/A31389	50

25 ^a Raw-starch digesting amylase, ^b Maltogenic α -amylase, ^c Maltotetraose-forming amylase (1,4- α -maltotetrahydrolase, ^d Maltopentaose-forming amylase, ^e thermostable α -amylase, ^f formerly *Clostridium thermosulfurogenes*.

AMYG, GAM and GLA : glucoamylase, AMY or AML : alpha-amylase, CGT : β -cyclodextrin glycosyltransferase or cyclomaltodextrin glucanotransferase, ORF : open reading frame

30 *A. : Aspergillus*, *B. : Bacillus*, *C. : Corticium*, *D. : Dictiostelium*, *H. grisea : Humicola grisea*, *H. resinae : Hormoconis resinae* (*Amorphotheca resinae*), *K. : Klebsiella*, *N. : Neurospora*, *S. : Streptomyces*, *Th. curvata : Thermomonospora curvata*, *Th.*

Thermoanaerobacter.

Table 4 - cont'd
References - cont'd

Table 4 References - cont'd

References Starch-binding Domains

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Table 5
Sources of Polysaccharide Binding Domains

	Binding Domain	Proteins Where Binding Domain is Found
5	Cellulose Binding Domains ¹	β -glucanases (avicelases, CMCase, cellodextrinases) exoglucanases or cellobiohydrolases cellulose binding proteins
10		xylanases mixed xylanases/glucanases esterases chitinases β -1,3-glucanases
15		β -1,3-(β -1,4)-glucanases (β -)mannanases β -glucosidases/galactosidases cellulose synthases (unconfirmed)
20	Starch/Maltodextrin Binding Domains	α -amylases ^{2,3} β -amylases ^{4,5} pullulanases glucoamylases ^{6,7} cyclodextrin glucotransferases ⁸⁻¹⁰ (cyclomaltodextrin glucanotransferases)
25		maltodextrin binding proteins ¹¹
	Dextran Binding Domains transferases ¹²	(<i>Streptococcal</i>) glycosyl
30		dextran sucrases (unconfirmed) <i>Clostridial</i> toxins ^{13,14} glucoamylases ⁶ dextran binding proteins
35	β -Glucan Binding Domains	β -1,3-glucanases ^{15,16} β -1,3-(β -1,4)-glucanases (unconfirmed) β -1,3-glucan binding protein ¹⁷
40	Chitin Binding Domains	chitinases chitobiases chitin binding proteins (see also cellulose binding domains) Heivein

45

¹Gilkes *et al.*, *Adv. Microbiol Reviews*, (1991) 303-315.²Søgaard *et al.*, *J. Biol. Chem.* (1993) 268:22480.

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Once the most appropriate polysaccharide binding moiety for a particular application has been identified, a PBP can be prepared by transforming into a host cell a DNA construct comprising DNA encoding the appropriate polysaccharide binding moiety. The phrase "polysaccharide binding peptide" intends an amino acid sequence which comprises at least a functional portion of the polysaccharide binding region of a polysaccharidase or a polysaccharide binding protein. By "functional portion" is intended an amino acid sequence which binds to an oligosaccharide polymer of interest.

25 The binding can be weak or strong depending upon the intended application. As an example, for promoting cell division, generally weak binding to a particular substrate is preferred; for promoting cell differentiation, generally strong binding to a particular substrate is preferred. Preferably, DNA encoding a protein of interest is ligated to the PBP DNA sequence. The fused gene

30 coding for the composition according to formula (1), or the PBP DNA sequence alone, is expressed in a host cell, either a eukaryotic or a prokaryotic cell. Where the PBP alone has been prepared, if desired, the expressed and isolated polysaccharide binding peptide can be conjugated to a compound of interest, *i.e.*, a growth factor or other moiety that stimulates or inhibits cell

35 proliferation or differentiation.

The techniques used in isolating polysaccharidase genes, such as a cellulase gene, and genes for polysaccharide binding proteins are known in the art, including

synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. (See, USPN 5,137,819, 5,202,247, and 5,340,731.) The sequences for several polypeptide binding domains, which bind to soluble oligosaccharides are known. (See, Figure 1.) The DNAs coding for a variety of polysaccharidases and
5 polysaccharide binding proteins also are known. Various techniques for manipulation of genes are well known, and include restriction, digestion, resection, ligation, *in vitro* mutagenesis, primer repair, employing linkers and adapters, and the like (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

10 The amino acid sequence of a polysaccharidase also can be used to design a probe to screen a cDNA or a genomic library prepared from mRNA or DNA from cells of interest as donor cells for a polysaccharidase gene or a polypeptide-binding protein gene. By using the polysaccharidase cDNA or binding protein cDNA or a fragment thereof as a hybridization probe, structurally related genes found in other
15 microorganisms can be cloned. Particularly contemplated is the isolation of genes from organisms that express polysaccharidase activity using oligonucleotide probes based on the nucleotide sequences of genes obtainable from an organism wherein the catalytic and binding domains of the polysaccharidase are discrete, although other polysaccharide binding proteins also can be used (see, for example, Shoseyov, *et al.*,
20 *Proc. Nat'l. Acad. Sci. (USA)* (1992) 89:3483-3487).

Probes developed using consensus sequences for the binding domain of a polysaccharidase or polysaccharide-binding protein are of particular interest. The β -1,4-glycanases from *C. fimi* characterized to date are endoglucanases A, B, C and D (CenA, CenB, CenC and CenD, respectively), exocellobiohydrolases A and B (CbhA
25 and CbhB, respectively), and xylanases A and D (Cex and XylD, respectively) (see Wong *et al.* (1986) *Gene*, 44:315; Meinke *et al.* (1991) *J. Bacteriol.*, 173:308; Coutinho *et al.*, (1991) *Mol. Microbiol.* 5:1221; Meinke *et al.*, (1993) *Bacteriol.*, 175:1910; Meinke *et al.*, (1994) *Mol. Microbiol.*, 12:413; Shen *et al.*, *J. Biol Chem* 266: (1991) 11335-11340; O'Neill *et al.*, (1986) *Gene*, 44:325; and Millward-Sadler *et al.*, (1994) *Mol. Microbiol.*, 11:375). All are modular proteins of varying degrees of
30 complexity (Fig. 1), but with two features in common: a catalytic domain (CD) and a cellulose-binding domain (CBD) which can function independently (see Millward-

Sadler *et al.*, (1994) *Mol. Microbiol.*, 11:375; Gilkes *et al.*, (1988) *J. Biol. Chem.*, 263:10401; Meinke *et al.*, (1991) *J. Bacteriol.*, 173:7126; and Coutinho *et al.*, (1992) *Mol. Microbiol.*, 6:1242). In four of the enzymes, CenB, CenD, CbhA and CbhB, fibronectin type III (Fn3) repeats separate the N-terminal CD from the C-terminal
5 CBD. The CDs of the enzymes come from six of the families of glycoside hydrolases (see Henrissat (1991) *Biochem. J.*, 280:309; and Henrissat *et al.*, (1993) *Biochem. J.*, 293:781); all of the enzymes have an N- or C-terminal CBD or CBDs (see Tomme *et al.*, (1995) *Adv. Microb. Physiol.*, 37: 1-81); CenC has tandem CBDs from family IV at its N-terminus; CenB and XylD each have a second, internal CBD from families III
10 and II, respectively. Cex and XylD are clearly xylanases; however, Cex, but not XylD, has low activity on cellulose. Nonetheless, like several other bacterial xylanases (see Gilbert *et al.*, (1993) *J. Gen. Microbiol.*, 139:187), they have CBDs. *C. fimi* probably produces other β -1,4-glycanases. Similar systems are produced by related bacteria (see Wilson (1992) *Crit. Rev. Biotechnol.*, 12:45; and Hazlewood *et al.*, (1992) *J. Appl. Bacteriol.*, 72:244). Unrelated bacteria also produce glycanases;
15 *Clostridium thermocellum*, for example, produces twenty or more β -1,4-glycanases (see Béguin *et al.*, (1992) *FEMS Microbiol. Lett.*, 100:523). For solid phase recovery systems, CBDs that bind insoluble polysaccharides are of particular use. For use in phase separation purification of growth-factor conjugates, a particularly useful CBD is
20 the binding domain of *C. fimi* endoglucanase C N1, which is the only protein known to bind soluble cellosaccharides and one of a small set of proteins that are known to bind any soluble polysaccharides.

Examples of a suitable binding domain are shown in Fig. 1, in which is presented an alignment of binding domains from various enzymes that bind to
25 polysaccharides; amino acid residues that are conserved among most or all of the enzymes are identified. This information is used to derive a suitable oligonucleotide probe using methods known to those of skill in the art. The probes can be considerably shorter than the entire sequence but should at least be 10, preferably at least 14, nucleotides in length. Longer oligonucleotides are useful, up to the full
30 length of the gene, preferably no more than 500, more preferably no more than 250, nucleotides in length. RNA or DNA probes can be used. In use, the probes are typically labeled in a detectable manner, for example, with ^{32}P , ^3H , biotin or avidin)

and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after the unhybridized probe has been separated from the hybridized probe. The hybridized probe is typically immobilized on a solid support such as nitrocellulose paper.

- 5 Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Although probes are normally used with a detectable label that allows easy identification, unlabeled oligonucleotides are also useful, both as precursors of labeled probes and for use in methods that provide for direct detection of double-stranded DNA (or DNA/RNA). Accordingly, the term "oligonucleotide
10 probe" refers to both labeled and unlabeled forms.

Generally, the binding domains identified by probing nucleic acids from an organism of interest will show at least about 40% identity (including as appropriate allowances for conservative substitutions, gaps for better alignment and the like) to the binding region or regions from which the probe was derived and will bind to a
15 soluble β -1,4 glucan with a K_a of $\geq 10^3 \text{ M}^{-1}$. More preferably, the binding domains will be at least about 60% identical, and most preferably at least about 70% identical to the binding region used to derive the probe. The percentage of identity will be greater among those amino acids that are conserved among polysaccharidase binding domains. Analyses of amino acid sequence comparisons can be performed using
20 programs in PC/Gene (IntelliGenetics, Inc.). PCLUSTAL can be used for multiple sequence alignment and generation of phylogenetic trees.

In order to isolate the PBP of a polysaccharidase or a polysaccharide binding domain from an enzyme or cluster enzyme that binds to a polysaccharide, several genetic approaches can be used. One method uses restriction enzymes to remove a
25 portion of the gene that codes for portions of the protein other than the PBP. The remaining gene fragments are fused in frame to obtain a mutated gene that encodes a truncated protein. Another method involves the use of exonucleases such as *Bal31* to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene deletion
30 methods result in a mutated gene encoding a shortened protein molecule which can then be evaluated for substrate or polysaccharide binding ability. Appropriate

substrates for evaluating and binding activity include those for the enzymes listed in Tables 1-5 above, as well as the carbohydrates listed in Table 6 below.

Growth-factor conjugates need not include an entire naturally-occurring growth factor or other cell modulating agent. It is sufficient that the conjugate include
5 a portion of the molecule that retains the desired biological activity. In the case of a polypeptide growth factor, for example, it is often advantageous to construct a growth-factor conjugate that does not include a transmembrane domain that is present in the native, naturally-occurring growth factor. The identification of such transmembrane domains, often by amino acid sequence analysis alone, is well known
10 to those of skill in the art, as are methods for obtaining truncated growth-factor molecules that lack the transmembrane or other undesired region. In the case of steel factor, for example, the 189 amino acid extracellular domain is of particular interest for stimulating cell proliferation. A steel factor conjugate can thus be constructed by deleting from the steel factor gene the portions that encode the 25 amino acid signal
15 peptide, the 23 amino acid hydrophobic membrane anchor, and the 36 amino acid cytoplasmic domain (*see* Huang *et al.* (1990) *Cell* 63:225-233; Flanagan *et al.* (1990) *Cell* 63:185-194; and Martin *et al.* (1990) *Cell* 63:203-211). Sequence and other structural analysis permits identification of the transmembrane and cytoplasmic domains of various growth factors for construction of conjugates by deletion of
20 transmembrane and cytoplasmic domains as well as the signal peptide for various growth factors including IL-2 (*see* Taniguchi *et al.* (1983) *Nature*, 302:305-310)), IL-3 (*see* Tentori *et al.* (1988) *J. Exp. Med.*, 168:1741-1747), IL-3 (*see* Yang, *et al.* (1986) *Cell*, 47: 3-10), interleukins that express B-cell stimulatory factor 1 and/or T-cell- and mast-cell-stimulating activities (*see* Lee, *et al.* (1986) *Proc. Natl. Acad. Sci. (USA)*,
25 83: 2061-2065); Yokota, *et al.* (1986) *Proc. Natl. Acad. Sci. (USA)*, 83:5894-5898), B-cell stimulatory factor-2 also termed IL-6 (BSF-2/IL-6) (*see* Yasukawa, *et al.* (1987) *EMBO J.*, 6:2939-2945), interleukin-9 (IL-9) (Modi, *et al.* (1991) *Cytogenet. Cell Genet.*, 57:114-116; human IL-9 gene (GenBank accession number M30135), interleukin-11 (IL-11) (McKinley, *et al.* (1992) *Genomics*, 13:814-819), interleukin-
30 12 (IL-12) (*see* Wolf, *et al.* (1991) *J. Immunol.*, 146:3074-3081) nerve growth factor (NGF) a neurotrophin and other neurotrophins, including neurotrophin-4 (*see* Ip, *et al.* (1992) *Proc. Natl. Acad. Sci. (USA)*, 89:3060-3064); sequences are available for

several species, including rat and human for all of them (GenBank accession numbers of the human neurotrophins are: NGF VO1511; BDNF:M61176; NT-3:M61180; and NT-4/5 M86528), erythropoietin (EPO) (*see* Lin, *et al.* (1985) *Proc. Natl. Acad. Sci.*, 82:7580-4), granulocyte colony-stimulating factor (GCSF) (*see* Nagata, (1986) *Nature*, 319:415-8), colony-stimulating factor-1 (CSF-1) (nucleotide sequences of CSF-1 c-DNAs cloned from mouse and man: GenBank accession numbers M21149, M21952, J03862 and M64592, M37435, respectively), g*** m*** colony-stimulating factor (*see* nucleotide sequences of GM-CSF cDNAs cloned from mouse and man: GenBank accession numbers X03020 and X03021), stem cell factor (SCF) (Martin, *et al.* (1990) *Cell*, 63:203-211), hepatocyte growth factor (HGF) (*see* Seki, *et al.* (1990) *Biochem. Biophys. Res. Commun.*, 172:321-327), epithelial growth factor (EGF) (*see* GenBank accession numbers for available sequences which follow: EGF: human: X04571; mouse: J00380; rat:M63585; TGF α : human: X70340; mouse: M9240; rat: M31076; amphiregulin: human: M30704; HB-EGF: human: M60278; monkey: M93012; mouse: L07264; rat: L05489; pig: X67295; betacellulin: mouse: L08394; *lin-3*: L11148; *spitz*: M95199; *gurken*: L22531; and pox viruses: vaccinia: J02421; Shope: M15921; myxoma: M15806 and M35234), and both platelet-derived growth factor A-chain (Betsholtz, *et al.* (1986) *Nature*, 320:695-9) and platelet-derived growth factor B-chain (Josephs, *et al.* (1984) *Science*, 223:487-91).

Once nucleotide sequences encoding the polysaccharide binding region and the growth-factor moiety have been identified, either as cDNA or chromosomal DNA, they can then be manipulated in a variety of ways to prepare a composition where the expression product has a structure represented by formula (1) above. The nucleotide sequence that codes for the polysaccharide binding region may be fused to a DNA sequence coding for a growth factor or a biologically active portion thereof. It is highly desirable that the three-dimensional structure of the component polypeptides be retained. Depending upon the source of the fragments and the length of the desired polypeptide, one or more restriction sites can be designed into the synthetic genes used to construct chimeric polypeptides. If possible, the restriction site(s) leaves the amino acid sequence of the polypeptide unaltered. However, in some case incorporation of a new restriction site(s) may yield an altered amino acid sequence without changing the activity of the protein.

During the construction of the expression cassette, various fragments of the DNA are usually cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation by joining or removing of sequences, linkers, or the like. Normally, the vectors are capable of
5 replication in at least a relatively high copy number in bacteria. A number of vectors are readily available for cloning in gram-negative bacteria, especially *E. coli*, including such vectors as pBR322, pTZ, pUC and the like. The cloning vectors are characterized by having an efficient replication system functional in the host bacterium.

10 The cloning vector generally has at least one unique restriction site, usually a plurality of unique restriction sites, and also can include multiple restriction sites. In addition, the cloning vector will have one or more markers which provide for selection of transformants. The markers normally provide resistance to cytotoxic agents such as antibiotics, heavy metals, toxins or the like, complementation of a
15 mutation that renders the host auxotrophic, or immunity to a phage. By appropriate restriction of the vector and the cassette, and, as appropriate, modification of the ends, by chewing back or filling in overhangs, to provide for blunt ends, by addition of linkers, by tailing, complementary ends are provided for ligation and joining of the vector to the expression cassette or component thereof.

20 After each manipulation of the DNA in the development of the cassette, the plasmid is isolated and, as required, the particular cassette component analyzed as to its sequence to insure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired fragment is excised from the plasmid and introduced into a different vector or the plasmid is restricted and the expression
25 cassette component manipulated, as appropriate. In some instances, a shuttle vector is employed which is capable of replication in different hosts requiring different replication systems. This may or may not require additional markers that are functional in the two hosts. Where such markers are required, these can be included in the vector so that the plasmid containing the cassette, two replication systems and the
30 marker(s) can be transferred from one host to another, as required. For selection, any useful marker may be used. Examples of suitable selectable markers include genes that confer resistance to ampicillin, tetracycline, hygromycin B, G418, and/or

neomycin and the like. However, although a marker for selection is highly desirable for convenience, other procedures for screening transformed cells are known to those skilled in the art, for example, transformed cells can be screened by the specific products they make; synthesis of the desired product may be determined by
5 immunological or enzymatic methods.

The DNA coding for the growth-factor conjugate is manipulated in a variety of ways to provide for expression. For example, the gene that codes for the PBP and the growth-factor moiety is operably linked to appropriate transcriptional and/or translational signals that are operable in the desired host organism. Expression vectors
10 can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, a HSV *tk* (thymidine kinase) promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.* (1986) *Immunol. Rev.* 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T Ag poly A addition
15 site), and transcriptional terminator sequences. Appropriate vectors for expressing growth-factor conjugates in insect cells usually are derived from the SF9 baculovirus. Illustrative transcriptional regulatory regions or promoters include, for bacteria, the *lac* promoter, the *trp* promoter, the *Tac* promoter (which is a hybrid of the *trp* and *lac* promoters); the lambda left and right promoters, and the like. The transcriptional
20 regulatory region can additionally include regulatory sequences which allow the time of expression of the fused gene to be modulated, for example, by the presence or absence of nutrients or expression products in the growth medium, temperature, etc. For example, expression of the fused gene can be regulated by temperature using a regulatory sequence comprising the bacteriophage lambda PL promoter, the
25 bacteriophage lambda OL operator and a temperature sensitive repressor. Regulation of the promoter is achieved through interaction between the repressor and the operator. A preferred promoter is the strong glucose-repression insensitive *Tac* promoter. Examples of high level expression vectors are described in Graham *et al.*, (1995) *Gene* 158:51-54.

30 Methods for synthesis of heterologous proteins in yeast are well known. *Methods in Yeast Genetics*, Sherman *et al.* ((1982) Cold Spring Harbor Laboratory Press) is a well recognized work describing the various methods available to produce

a growth-factor conjugate in yeast. Suitable vectors for expression in yeast usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired. Suitable vectors include those described in the
5 literature (see, for example, Botstein *et al.* (1979) *Gene* 8:17-24; Broach *et al.* (1979) *Gene* 8:121-133). Expression vectors that are suitable for use in various eukaryotic host cells are produced by several commercial manufacturers of biological reagents (see, e.g., product catalogs from Stratagene Cloning Systems, La Jolla, CA; Clontech Laboratories, Palo Alto, CA; Promega Corporation, Madison WI).

10 The expression cassette can be included within a replication system for episomal maintenance in an appropriate cellular host or can be provided without a replication system, in which the vector can become integrated into the host genome. The DNA can be introduced into the host in accordance with known techniques, such as transformation, using calcium phosphate-precipitated DNA, transfection by
15 contacting the cells with a virus, microinjection of the DNA into cells or the like.

Suitable host organisms include microbes such as prokaryotes, including *E. coli*, *Streptomyces*, and *Bacillus*, and eukaryotes such as the yeasts *Saccharomyces* (esp. *S. cerevisiae*) and *Pichia pastoris*. Mammalian and other higher eukaryotic cells also are useful for expression of the growth-factor conjugates. A number of suitable
20 mammalian host cell lines which can express the growth-factor conjugates have been developed in the art, and include the HEK293, BHK21, and CHO cell lines, and various human cells such as COS cell lines; HeLa cells, myeloma cell lines, Jurkat cells, and the like. Insect cells are another eukaryotic system that is useful for expressing the growth-factor conjugates. Appropriate vectors for expressing growth-
25 factor conjugates in insect cells usually are derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (*see*, Schneider (1987) *Embryol. Exp. Morphol.* 27:353-365).

Once the DNA encoding the PBP-growth-factor conjugate has been introduced
30 into the appropriate host, the host can be grown to express the conjugate. The recombinant products can be glycosylated or non-glycosylated, having the wild-type or other glycosylation. The amount of glycosylation depends in part upon the

sequence of the particular peptide, as well as the organism in which it is produced. Thus, expression of the product in *E. coli* cells results in an unglycosylated product, and expression of the product in insect cells generally results in less glycosylation than expression of the product in mammalian cells. Expression in yeast can result in
5 hyperglycosylation.

For isolation of the PBP-growth-factor conjugate, where the product is retained in the host cell, the cells are harvested, lysed and the product isolated and purified using methods known to those of skill in the art. In some instances, it can be desirable to provide for a signal sequence (secretory leader) upstream of and in
10 reading frame with the structural gene, which provides for secretion of the growth-factor conjugate. Illustrative secretory leaders include the secretory leaders of penicillinase, immunoglobulins, T-cell receptors, outer membrane proteins, and the like. By fusion in proper reading frame the growth-factor conjugate can be secreted into the medium or the periplasmic space of bacteria. In bacterial expression systems
15 such as *E. coli*, a significant fraction leaks into the extracellular media (Ong *et al.*, *Biotech. Bioeng.* (1993) 42:401). Where the product is secreted, the nutrient medium can be collected and the product isolated using procedures known to those of skill in the art. To produce an active protein it can be necessary to allow the protein to refold.

In a preferred embodiment, the PBP-growth-factor conjugate is purified by
20 affinity chromatography. The substrate binding region is bound to an appropriate substrate, or a different affinity tag that is incorporated into the growth-factor conjugate used. For example, the PBP-growth-factor conjugate can be constructed so as to include an affinity tag such as hexahistidine, Streptavidin, or GST; such affinity tags are useful for affinity purification using, *e.g.*, nickel-Sepharose, biotin, and
25 glutathione, respectively.

The growth-factor conjugates of the invention generally are immobilized on a substrate of the polysaccharidase binding peptide. By "immobilized" is meant that the PBP-growth-factor conjugates are bound to suitable substrates, either insoluble or soluble, via the PBP. Substrates of interest include, for example, insoluble
30 polysaccharides such as: cellulose, a polysaccharide composed of D-glucopyranose units joined by β -1,4-glycosidic linkages and its esters, *e.g.* cellulose acetate; xylan, in which the repeating backbone unit is β -1,4-D-xylopyranose; chitin, which resembles

cellulose in that it is composed of β -1,4-linked N-acetyl, 2-amino-2-deoxy- β -D-glucopyranose units. Other oligosaccharides that are of use include α -1,4-glycans such as starch. Enzymes that are capable of binding to polysaccharides, such as those listed above, are of interest in the subject invention as a source of amino acid

5 sequences capable of binding to such substrates. The substrates are useful in forms that include microcrystalline cellulose (bacterial, cellex mx (BioRad), Avicel), cotton, paper, hollow cellulose fibers, microcarriers (*e.g.*, Cellsnow™, Kirin Brewing Co., Japan) sponges, wound coverings, and the like. The substrate can optionally be reversibly or irreversibly bound to a solid support or can itself comprise a solid

10 support (*e.g.*, cotton fabric, paper, cellulose hollow fiber, growth chamber, microcarrier, etc.). The conjugates also can be immobilized on the surface of any of a variety of apparatus, which has been coated with a polysaccharide. As an example, sheets of cellulose can be prepared from the cell walls of the marine alga *Valoria ventricosa* and dried onto the surface of the apparatus. The term immobilized growth

15 factor includes the PBP tag used for immobilizing the growth factor on a polysaccharide substrate, with or without a cleavable linker between the growth factor and the tag.

It is a theory of the invention that modulation of cell division occurs by way of transient activation of a growth-factor receptor, including dimerization of the ligand

20 receptor complexes in the cell membrane, and internalization of the ligand receptor complex. Therefore, in order to modulate cell division using an immobilized growth factor, the growth factor is presented in a format that will allow the appropriate movement of the receptor-growth hormone complex in the cell membrane to form dimers or other larger structures and to permit internalization of the receptor-growth

25 hormone complex. This can be accomplished in several ways. For example, a growth factor can be immobilized on a structure that can be internalized by a cell together with the receptor-growth-factor complex that is bound to it. In another approach, a growth factor can be coupled to an immobilizing surface, generally at a low concentration per unit area using a surface to which the binding is of a sufficiently

30 low affinity that the growth factor is removed from the surface subsequent to binding to the receptor. The surface can be reused until all the growth factor has been scavenged, generally at least 3 to 5 times. For example surfaces for the practice of the

invention include laboratory glassware, such as tissue culture plates and flasks which can be coated with cellulose acetate, which is readily available. Because the backing of photographic film is also made of cellulose acetate, patternable cellulose acetate surface may be generated by exposing photographic film to a pattern to yield exposed
5 cellulose acetate in the same pattern after development. More intricate patterning may be effected by patterning photoresist onto the film before exposing the film to the pattern. Still more intricate patterning may be effected by combining the preceding with other photolithographic and even electron beam techniques for even more intricate patterns with smaller feature sizes. X-ray film may be exposed to a pattern to
10 create a cellulose acetate pattern onto which cytokine fusion proteins may be attached. Cells exposed to such a patterned surface would bind to it in the predetermined pattern. Such patterning of cell growth might be utilized to pattern cell growth in a manner that simulates the histology of an organ such as the liver. Also neuronal attachment to surfaces can be patterned for example to guide neural growth and
15 attachment to certain specific synapses in therapy for spinal or other injury. X-ray photoelectrons can be focused to yield nanometer feature dimensions on X-ray film.

The growth factor also can be immobilized incorporating a cleavable linker molecule between the growth factor and the PBP. The linker molecule generally is one which can be cleaved without damaging the cells, such as a linker molecule which
20 can be cleaved by a protease. This method can be used to maintain growth-factor dependent cells until such time as it is desirable to expand the cell population, at which time the culture is treated with a protease to cleave the linker molecule. Examples of cleavable linkers that can be used include polyalanine and polylysine, the IEGR (SEQ ID No: 3) amino acid sequence cleaved by Factor Xa, or any amino acid
25 sequence which is susceptible to cleavage at a specific site in the sequence or non-specifically. Specific or non-specific proteases that may be utilized include Factor Xa, trypsin, papain, and various viral proteases. Chemical agents which may be utilized to cleave linker sequences include cyanogen bromide. Examples of growth factors that may be utilized for cell maintenance include EGF, NGF, stem cell factor,
30 an insulin-like growth factor, an angiogenesis factor, a blood forming factor, FLT 3 ligand, steel factor, IL-2, IL-3, GCSF, GMCSF, MCSF, EPO, a nerve cell growth factor, a chondrogenic factor, and an osteogenesis factor. A combination of factors,

some with the linker, and some without might be employed to modulate cell division and differentiation, and such a combination may better mimic the in vivo growth and development of cells, which may be subjected to internalizable and non-internalizable factors simultaneously. For example, a mixture of EGF-CBD, having a linker, and

5 NGF-CBD with no linker on a surface may be utilized to create a transient stimulation of proliferation combined with a sustained differentiation signal to neuronal cells. Patterning techniques may permit spatial localization of the transient proliferation and sustained differentiation signals. Also, triple fusion proteins comprising, for example, CBD-NGF-cleavable linker-EGF may be employed to attract neurons to a surface,

10 transiently stimulate proliferation by cleaving the EGF to provide an initial transient signal for proliferation, followed by a sustained differentiation signal. Other sequential cell modulations might be effected for neurons and other cells by utilizing fusion proteins having more than one growth factor linked in the appropriate manner to CBD or another PBD by the appropriate linker or linkers.

15 These immobilized growth factors also can be used, for example, in conjunction with a method for purifying a cell population, and/or removing contamination cells from the cell population, such as, for example, removing cancer cells from bone marrow cells prior to reimplantation into a patient. When a fusion protein of this invention is immobilized onto a porous surface that has pore

20 dimensions large enough to permit cells to pass if they do not bind the fusion protein, different cell populations may be separated. For example, non-internalizable cellulose beads, large cellulose crystals, or cellulose based textiles and the appropriate fusion protein might be used to separate cells. A separation of primitive hematopoietic stem cells from leukemic B and T cells may be effected by binding SCF-CBD to gauze and

25 passing a patients blood through the gauze. Differentiated B and T cells do not express the *kit* receptor for SLF, and would therefore pass through the gauze, while stem cells which express the *kit* receptor would be captured for return to the patient in conjunction with or instead of bone marrow replacement therapy. Utilization of other growth factor receptors may be utilized for different separations. For example FLT 3

30 Ligand is expressed by primitive stem cells, and therefore might be utilized for separating stem cells from differentiated cells, or for separating the more primitive of stem cells from a collection of stem cells. Additionally, immobilized cleavably linked

SCF-CBD may be employed to temporarily arrest the division of intermediate stem cells; more mature or less mature stem cells, or both, may be killed by adding IL-3 and 5-FU, FLT 3 and 5FU, or IL-3 and FLT 3 and 5-FU respectively. After the unwanted stem cells have been eliminated, the linker may be cleaved and to permit
5 the intermediate stem cells to proliferate for reimplantation, transplantation or culturing.

The immobilized growth factors also find use for *ex vivo* differentiation and/or maintenance of growth-factor dependent cells, particularly cells of the hematopoietic system such as stem cells, as well as megakaryocytes and T-lymphocytes. For
10 example, the methods are useful for cultivation of components of bone marrow such as pre-CFU-S, BFU-E, MK, CFU-MK, CFU-GEMM and GM. The methods involve growing cells responsive to the growth factor in contact with the immobilized growth factor. Since the immobilized factors generally are not consumed by the cell cultures, the factors provide a continuous localized stimulus for cell activation and/or
15 differentiation. The cell cultures can be in culture plates, growth chambers, and the like, particularly perfusion cultures in which growth medium is continuously added and removed to allow long-term cell maintenance and/or large-scale use of a cell population, particularly transgenic cells including commercially valuable proteins. For production of cellular components, cells that can be cultured in perfusion culture
20 include specific effector T cells, stem cells obtained from bone marrow or blood, megakaryocytes or other hematopoietic cells, and the like.

The growth factors also can be bound via the PBP to an extracorporeal device that contains a substrate for the PBP, *e.g.*, a paper filter or a hollow fiber, and the device used for the *ex vivo* manipulation of growth-factor dependent cells. For
25 example, blood from a patient in need of expansion, activation, or differentiation of a particular growth-factor dependent cell type can be passed through an extracorporeal device within which a growth-factor conjugate is immobilized. In one embodiment, the growth-factor conjugate is bound to a cellulose matrix such as a hollow fiber that is present within the extracorporeal device. Growth-factor dependent cells that are
30 passed through the fiber thus contact the immobilized growth-factor moiety, which captures the cognate cells by receptor binding and modulates proliferation, activation, or differentiation of the cells, depending upon intended use of the cells, and based

upon the growth factor(s) used and their mode of attachment to the fiber. An example for which this method is useful is the activation of anti-tumor T cells by passing a cancer patient's blood through an extracorporeal device that contains a polysaccharide to which is bound a growth-factor conjugate that comprises interleukin-2. This method may be employed in conjunction with a hollow fiber cartridge hemodialysis system having interleukin-2-PBD bound to the fibers.

The growth-factor conjugates also can be used for enhancing wound healing by contacting a wound with a growth-factor conjugate, generally one that is bound to a polysaccharide substrate to which the conjugate binds, such as cotton, for example in a bandage. The growth-factor moiety used in the claimed methods is one that is chemotactic for cells involved in wound healing. Alternatively, the growth factor can stimulate proliferation of cells involved in wound healing, or inhibit proliferation of cells to prevent or minimize scar tissue formation. The growth-factor conjugate is administered to the wound site in an amount effective to enhance cell migration to the wound site and/or modulation of target cell growth. The dressing may be used internally or externally, according to the same principles. Internally used dressings may be permanent or degraded during healing. For example, either permanent or absorbed sutures may be coated with immobilized growth factors according to the invention. Other intracorporeal uses include using an immobilized cytokine on cellulose as a vaccine adjuvant, and the timed release of therapeutic fusion protein temporarily immobilized on a cellulose product approved for internal use, such as AQUATERIC™ (FMC Corp.), which is contacted with a cellulase to effect the release of the therapeutic fusion protein.

Immobilized growth factors may be utilized in making implants, including bone implants. For example, porous cellulose may be fashioned into a desired form and SCF-CBD and EGF-CBD may be bound to the cellulose matrix. The porous cellulose having cytokines on its surface may then be contacted with a culture of the patients explanted bone marrow. Some bone marrow cells would be attracted into the porous cellulose matrix and attach to the surface. Such an implant would be similar to bone, and generate immune and red blood cells.

Additionally components of artificial organs and other implants may be made more biocompatible and better integrated into the tissues that surround them. For

example, indwelling catheters and hoses are often required for various medical treatments. Some artificial hearts require indwelling air hoses to power the pump. Infectious agents which enter at the site the integument is breached by the hose are the usual cause of death in these patients. The breach in the skin may be sealed by
5 employing a porous cellulose plug which fits around the hose. EGF-CBD may be immobilized on the cellulose surfaces and the plug is then contacted with the patients bone marrow and skin cells. Skin healing around such a plug after implantation will intercalate with the plug, creating an entry for the hose that will not allow bacteria to enter freely. Additionally patterning, as on film, employing conventional
10 photolithographic and perhaps electron beam methods could be used to structure cell growth to simulate functionally important tissue histology, to simulate organ parts for implants. Such implants could be used as tissue replacers and as a reservoir for making biological molecules, including proteins. The cells used for such implants may be engineered or naturally occurring, and they may originate from the recipient,
15 another individual, or even another species depending upon whether immunoisolation is possible and practicable. One example would be to modify fibroblasts or other appropriate cells from an insulin dependent diabetic to express insulin under the control of a glucose-induced promoter; such cells would be most useful if immobilized onto a matrix which is exposed to portal blood, where glucose levels rise
20 fast and high after a meal.

Other therapeutic applications include the enhancement of nerve regeneration. For example, a conjugate that includes a neurotrophic factor and/or nerve growth factor is immobilized on a polysaccharide substrate such as a membrane via the polysaccharide binding peptide moiety of the conjugate and applied to nerve tissues
25 damaged by injury or illness (*see e.g.* USPN 5,229,500). The immobilized conjugate is then placed in the proximity of the damaged nerve for a sufficient time to result in partial or total regeneration of the nerve. Again patterning is important; "re-connecting" severed spinal nerves requires both stimulation of growth and precise guiding each axon to the correct place.

30 The claimed methods are also useful for cultivating factor dependent cells that require specific local concentrations or gradients of factors, such as nerve cells. A gradient is generated by methods known to those of skill in the art. For example, flow

techniques can be used to generate a gradient by contacting a polysaccharide matrix with a solution of PBP-growth-factor conjugate. Proximal regions of the matrix absorb the highest amount of conjugate from the flow stream. Alternatively, an agar gel can be impregnated with growth factor conjugate and placed on the polysaccharide growth matrix (cellulose). Growth factor is transferred from the gel to the surface by diffusion or electroporation. The gel can be shaped to provide any gradient pattern, e.g., a wedge shaped agar gel provides a linear gradient to the polysaccharide matrix.

The growth-factor conjugates can be used to obtain a population of cells that is enriched in cells that are dependent upon a particular growth factor or other moiety for proliferation or differentiation. Cells that carry a cell surface receptor for the growth factor are contacted with a growth-factor conjugate that is immobilized on a substrate for the polysaccharidase from which the substrate binding domain was derived. If an insoluble substrate is used, undesired cells are removed from the immobilized growth-factor dependent cells by washing.

The methods for concentrating growth-factor dependent cell types and/or receptors can be performed in batch mode, or by passing a cell suspension through a column. For example, the growth-factor conjugates can be bound to a polysaccharide matrix contained within an affinity chromatography column or other appropriate purification system. The conjugate is contacted with a sample mixture that contains a ligand that binds to the growth-factor moiety, including ligands that are cell-bound, under ionic conditions that allow binding of the ligand to the growth-factor conjugate. Unbound molecules and/or cells are then removed by washing the matrix. The bound molecules and/or cells are then isolated by washing the matrix with a buffer that elutes the bound molecules. For example SLF-CBD may be used to separate bone marrow putative cells from stromal and other support cells, and for separating healthy hematopoietic stem cells from more differentiated leukemic cells. And, immobilized IL-2 as for example interleukin-CBD bound to a cellulose matrix may be utilized to separate B cells from mammalian blood for monoclonal antibody production. Immobilized IL-2 and IL-3 may be utilized for separating T-cells from HIV patients. Various CD-CBD and ICAM-CBD constructs, including CD44-CBD may be utilized to separate and concentrate various cell populations.

For some applications, it is desirable to release the immobilized cells after the concentration step. Several methods can be used to remove immobilized growth-factor conjugates and/or attached cells from a polysaccharide substrate polymer. For example, PBP compounds bind specifically and strongly to the oligosaccharide
5 polymer but can be removed easily by elution with a low ionic strength solution (such as water), or a high pH solution a chaotropic salt. These methods can release the entire conjugate from the oligosaccharide polymer, together with any attached cell and/or receptor. The temperature for desorption is not critical and generally in the range of 10°C-40°C, although ambient temperatures are generally preferred, *i.e.*,
10 about 20°C. Physiologically compatible conditions are used when viable cells are desired. For example, a low ionic strength, physiological pH solution that lacks chaotropic salts can be used to release cells from a substrate.

If viability of cells attached to the growth-factor conjugate is not of concern, for example, where the receptor for the growth factor itself or the growth-factor
15 receptor pair is being purified, a pH 9.5 carbonate buffer or 6M guanidine HCl can be used for this desorption step. Dilute sodium hydroxide (about 0.1M) can be used in some cases. The nature of the PBP can be modified to alter its adherence properties so that it can, or, if desired, cannot, be desorbed by water. Application of the desorption medium to the matrix causes release of the conjugate from the
20 oligosaccharide polymer. For isolation of the PBP-conjugate and associated cells following release from the substrate, various techniques can be used. For example, the polysaccharide surface can be washed free of the PBP-conjugate with the desorption solution as described above. The PBP-conjugate then is separated from the desorption solution, for example, by changing its ionic strength or pH and readsorbing
25 the PBP-conjugate on an ion exchange medium or on a second polysaccharide matrix.

Alternatively, cells or other ligands immobilized on a polysaccharide substrate can be released from the substrate by cleaving the growth-factor conjugate by proteolysis using either a nonspecific general protease such as proteinase K or trypsin, or a specific protease. A non-specific protease can be used to completely degrade the
30 PBP portion of the PBP complex, thus releasing it from the oligosaccharide polymer. For example, release can be effected by treatment by proteinase K at a concentration of about 50 µg/ml for about 20 minutes at about 37°C. Din *et al.* (1991)

Bio/Technology, 9:1096-1099. A specific protease can also be used to release bound compounds and/or cells from a polysaccharide substrate to which they are bound. For example, one can include a protease recognition site or a chemical cleavage site between the growth-factor moiety and the PBP. The PBP remains bound to the
5 oligosaccharide polymer. Examples of recognition sites include those for collagenase, thrombin, enterokinase, and Factor X_a which are cleaved specifically by the respective enzymes. Suitable expression systems for Factor X_a and for Factor X_a-CBD fusion proteins have been developed (*see* Assouline *et al.* (1993) *Protein Eng.*, 7:787). One of the two native isoforms of steel factor (KL-1) contains a protease cleavage site
10 within its extracellular domain (Huang *et al.* (1992) *Mol. Cell. Biol.* 3:349-362; Pandiella *et al.* (1992) *J. Biol. Chem.* 267:24028-24033); cleavage at this site by a protease is thus a means for releasing the steel factor moiety of a growth-factor conjugate from a PBP. Chemical cleavage sites sensitive, for example, to low pH or cyanogen bromide, can also be used. The PBD thus provides a means of attaching
15 cells to the oligosaccharide polymer, which cells later can be removed.

Where cleavage is used, the growth-factor moiety and attached ligand and/or cell can be cleaved readily from the polysaccharide binding region by the use of a protease specific for a sequence present between the polysaccharide binding region and the growth-factor moiety leaving the PBP bound to the oligosaccharide polymer.
20 Preferably, the protease is provided in a form which will facilitate its removal following cleavage of the growth-factor moiety from the PBP. As an example, the cleavage protease can be prepared as a cleavage enzyme complex, wherein the protease is bound to a second polysaccharide binding moiety having a substrate specificity different from that of the first polysaccharide binding moiety bound to the
25 polypeptide of interest and/or having different binding characteristics (Assouline *et al.* (1993) *supra*; Assouline *et al.* (1995) *Biotechnol. Prog.* 11:45-49). Thus, cleavage of the binding domain from the recombinant protein of interest can be done in solution and the cleavage enzyme complex then removed by binding to a polysaccharide substrate to which the first polysaccharide binding moiety does not bind.
30 Alternatively, the cleavage enzyme complex can be immobilized on a polysaccharide matrix to which the first polysaccharide binding moiety does not bind. (*See* Assouline *et al.* (1993) *supra*; Assouline *et al.* (1995) *supra*). The purified cells or growth-factor

moiety are released from the oligosaccharide polymer free of contaminating PBPs which remain bound to the polymer.

The following examples are offered by way of illustration and not by way of limitation.

5

EXAMPLES

Abbreviations

*p*NPC = *p*-nitrophenyl- β -D-cellobioside;

HPA = hide powder axure;

10 gCenA and gCex = the glycosylated forms of CenA and Cex from *C. fimi*;

ngCenA and ngCex = the non-glycosylated forms of CenA and Cex from recombinant *E. coli*;

RPC = reverse-phase chromatography;

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

15 α -Pro/Thr = rabbit antiserum directed against synthetic Cex Pro/Thr box;

PMSF = phenyl-methylsulfonyl fluoride.

SLF protein = steel factor

Biological Culture Deposits

20 The following deposits have been made with the American Type Culture Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland 20852. A derivative of the cloned gene *CenA* on plasmid pcEC-2 in *Escherichia coli* C600 was deposited on April 23, 1986 and given ATCC Accession No. 67101. A derivative of the cloned gene *Cex* on plasmid pEC-1 was deposited on May 27, 1986 and given

25 ATCC Accession No. 67120. *E. coli* JM83, pUC12-1.1*cex* was deposited on April 23, 1986 and given ATCC Accession No. 67102. The full nucleotide sequences of pTugA (Accession Number L24193), pTugAS (Accession Number L24367), *C. fimi* CenA (Accession Number M15823), and *C. fimi* CenC (Accession Number X57858) have been deposited with GenBank.

30

Example 1

Construction of a Fusion Gene Encoding Steel Factor and a Cellulase Binding Domain

5 The fusion protein SLF-CBD comprises the extracellular domain of steel factor (Fig. 1A) linked to the cellulose binding domain of the *Cellulomonas fimi* exoglucanase Cex (Fig. 1B) to create a fusion between steel factor (SLF) and the cellulose binding domain of Cex (CBD_{Cex}), designated as (Fig. 1C). In the native Cex enzyme the catalytic domain is separated from the binding domain by a PT linker
10 which includes a series of repeating proline-threonine units. This linker was included in the SLF-CBD construct to separate the steel factor domain and the cellulose binding domain. A Factor Xa proteolytic cleavage site was introduced between the two domains, upstream of the PT linker, to facilitate the removal of the CBD if necessary. A hexahistidine affinity tag was added to the amino terminus of the
15 protein so that it could be purified either via the CBD using cellulose (Greenwood *et al.* (1990) *Cell* 63: 203-211) or via the hexahistidine affinity tag using nickel-Sepharose (Laemmli (1970) *Nature* 227: 680-685) as a matrix. When properly processed with the removal of the Cex signal peptide, SLF-CBD has a predicted molecular weight of 34.1 kDa. Enzymes and buffers were purchased from GIBCO
20 BRL (Grand Island, NY). All genetic manipulations were carried out in the *Escherichia coli* (*E. coli*) strain DH5a (Hanahan (1983) *J. Mol. Biol.* 166: 557-580).

 A gene that encodes the SLF-CBD fusion protein was constructed as follows. The coding sequence for the signal peptide from the cellulase Cex (O'Neill *et al.* (1986) *Gene* 44: 331-335) was introduced into the expression plasmid pTUG AS
25 (Fig. 2) using site directed mutagenesis (Zoller and Smith (1982) *Nucleic Acids Res.* 10: 6487-6500). Coding sequences for a hexahistidine affinity tag followed by an *NheI* restriction site were introduced in frame at the 3' end of the Cex signal sequence coding region. The resulting fragment was subcloned into the non-expression plasmid pSL1180 (Pharmacia Ltd., Piscataway, NJ), as an *NcoI*-*Hind* III fragment. A gene
30 fragment that encodes the extracellular domain of murine steel factor (Anderson *et al.* (1990) *Cell* 63:235-243; GenBank Accession No. M38436) was modified by polymerase chain reaction (PCR) (Kaufman and Evans (1990) *BioTechniques* 9:

304-306) to introduce an *Xba*I restriction site onto the 5' end of the gene fragment and *Stu*I and a *Hind* III sites onto the 3' end of the gene. This product was purified from an agarose gel using a QIAEX gel extraction kit (QIAGEN Ltd., Chatsworth, CA) and inserted downstream of the Cex signal peptide after cleavage of the Cex signal peptide-containing plasmid with *Nhe*I and *Hind*III. DNA from the original SLF gene was exchanged for the PCR product between two unique restriction sites which encompassed more than 90% of the gene, and the remaining vector junctions and flanking DNA were sequenced. The gene sequence encoding the cellulose binding domain from Cex, as well as its proline-threonine linker was then excised as a *Stu*I-*Hind*III fragment from the plasmid pUC12-1.1 Cex (PTIS) (ATCC Accession Number 67102 and see USPN 5,340,731) and inserted, in frame, downstream of the gene encoding the steel factor extracellular domain. The entire construct was then excised from pSL1180 as an *Nco*I-*Hind*III fragment and inserted into the high expression level plasmid pTug AS (Fig. 3) which had previously been modified to include kanamycin resistance by the insertion of a cassette (TN5). This plasmid was designated as pSLF/CBD 1.0.

Example 2

Expression and Purification of SLF-CBD

The plasmid pSLF/CBD 1.0 was transformed into *E. coli* JM101 (Yanisch-Perron *et al.* (1985) *Gene* 33: 103-119) for expression. Several colonies were scraped from a plate and used to inoculate a 5 ml test tube of Terrific Broth (Tartof and Hobbs, *Bethesda Res. Lab. Focus* 9: 12) containing 50 mg/ml kanamycin. This tube was incubated with shaking at 37°C for 4 hours and then used to inoculate four flasks containing 500 ml of the same medium at 0.2% volume. These flasks were then incubated at 37 C with shaking at 250 rpm until an optical density (OD₅₅₀) of 2 was reached. Production of the fusion protein by cells was induced by the addition of 0.1 mM IPTG and shifted to 30°C with shaking at 100 rpm for a further 8 hours. The cells were then harvested and the periplasmic proteins isolated by osmotic shock (Neu and Heppel (1965) *J. Biol. Chem.* 240: 3685-3692). Five mg of Avicel microcrystalline cellulose (FMC International, Cork, Ireland) was added to either 10

ml of culture supernatant or 1 ml of periplasmic extract and then removed by centrifugation. SDS-PAGE loading buffer was then added to the Avicel, which was then boiled for two minutes and loaded directly onto a gel for SDS-PAGE analysis (Laemmli, *supra.*) (Fig. 4A).

5 The periplasmic extract was buffered to a pH of 8.0 using 50 mM Tris-base (Boehringer-Mannheim, Indianapolis, IN) and concentrated to a volume of 2 ml by ultrafiltration through a 10 kDa pore size membrane. The sample was then purified using metal chelate affinity chromatography (MCAC) (Hochuli (1990) In *Genetic Engineering, Principles and Practice*, Vol. 12 (J. Setlow, Ed.), pp. 87-98, Plenum,
10 NY) using a 20 ml column packed with His-Bind resin (Novagen Inc., Madison, WI, cat. no. 69670) with elution of the protein at between 100 mM and 200 mM imidazole. The peak fractions were pooled and the elution buffer was exchanged for phosphate buffered saline (PBS) and the sample concentrated again using ultrafiltration. Purity of the final sample was evaluated by SDS-PAGE analysis and the
15 protein concentration determined by absorbance at 280 nm wavelength (Scopes (1974) *Anal. Biochem.* 59: 277-282) and by the Bradford method (Bradford (1976) *Anal. Biochem.* 72: 248-254). SLF-CBD released into the culture supernatant was purified in the same way except for the addition of an initial precipitation step to reduce the volume of the sample as follows. The supernatant was chilled to 0 C and
20 brought to 80% (w/v) of saturation ammonium sulfate while maintaining the pH at 6.05 (the predicted isoelectric point of the fusion protein) by the addition of 1N NH_4OH . The precipitated protein was collected by centrifugation at 10 000g and resuspended in MCAC loading buffer. The protein was analyzed by both SDS-PAGE and by western blotting (Burnette (1981) *Anal. Biochem.* 112: 195-203) using either
25 anti-murine steel factor polyclonal neutralizing antibodies (R&D systems, Minneapolis, MN, cat. no. AB-455-NA) or anti-CBD antibodies (more precisely rabbit anti-CEX antibodies generated by Emily Kwan, Cellulase Group, University of British Columbia).

 In cultures of *E. coli* JM101 (Ong *et al.* (1993) *Biotechnol. Bioeng.* 42:
30 401-409) transformed with pTK-SLF-CBD, the SLF-CBD fusion protein was present in both the periplasm and the culture supernatant, and could be recovered from either source by binding to Avicel (Fig. 4A). Although SLF-CBD can be purified by

affinity chromatography on Avicel, binding of the fusion protein to cellulose was strong and difficult to reverse without denaturing the SLF-CBD. It was more convenient to use the amino terminal histidine affinity tag for purification.

SLF-CBD from the periplasm was purified directly by high-performance liquid chromatography using a column packed with a nickel-Sepharose resin (Fig. 4B). The protein eluted as a single peak from this column at an imidazole concentration between 100 mM and 200 mM. SLF-CBD in the conditioned growth medium was purified in the same way following an initial volume reduction by ultrafiltration. A final yield of 0.7 mg/L of purified protein was obtained from the periplasm, and 1.8 mg/L from the supernatant. Amino-terminal sequence analysis of this protein confirmed that the signal peptide had been removed; and western blotting and SDS-PAGE analysis were carried out to confirm the identity of the protein (Fig. 5).

15

Example 3

Stimulation of Cell Proliferation using Immobilized SLF-CBD

Analysis of stimulatory activity of soluble and immobilized SCF-CBD

Purified SCF-CBD diluted in hybridoma serum-free medium (H-SFM, Gibco BRL, Grand Island, NY) was mixed with 1 µg bacterial microcrystalline cellulose (BMCC, 8) mL⁻¹ in the wells of 96 well tissue culture plates (Costar corp. Cambridge, MA). After 12 h incubation at 37°C in an atmosphere of 5% CO₂ balance air, 2x10⁴ B6SutA cells in H-SFM were added to the mixture for a final volume of 170 µl. After incubation of the cultures for 48 hours, biological activity was measured using MTT. Values are the average of duplicates ± standards error.

25

Bacterial microcrystalline cellulose (BMCC) was prepared as described in Gilkes *et al.* (1992) *J. Biol. Chem.* 276: 6743-6749), sterilized, and resuspended at various concentrations in hybridoma serum free medium (H-SFM) (GIBCO BRL, Grand Island, NY, Cat. No. 12045-019).

30

Cell proliferation assay

Factor-dependent cell lines were grown to late log phase (three days in the case of MO7e and TF-1 cells, and two days in the case of B6SUtA cells), washed three times in H-SFM, diluted 1:4 and added to liquid cultures. Cells were then
5 allowed to proliferate with or without $1 \mu\text{g BMCC mL}^{-1}$ for 48 hours at $37^{\circ}\text{C}/5\% \text{CO}_2$. Viable cells were determined by trypan blue exclusion in hemocytometric counts. Values are the average of duplicates \pm standard error. Non-viable cells constituted less than 5% of the total cell number in all cases and were not included in statistical analysis.

10 Samples of purified SLF-CBD, obtained as described in Example 2, were diluted in H-SFM. Twenty μl protein test samples were placed into wells in sterile 96 well tissue culture plates (Costar corp. Cambridge, MA, Cat. No. 3595). In experiments where BMCC was used, $50 \mu\text{l}$ of a BMCC suspension in H-SFM was added to each well, and in experiments where no BMCC was used $50 \mu\text{l}$ of H-SFM
15 alone was added. SLF-CBD and BMCC then were incubated together for 12 hours at 37°C with $5\% \text{CO}_2$ before the addition of $100 \mu\text{l}$ of B6SUtA cells (Greenberger et al. (1983) *Proc. Nat'l Acad. Sci. USA* 80: 2931-2935) which had been grown to one day past confluence and then resuspended in H-SFM at a concentration of 5×10^5 cells/ml, to give a final assay volume of $170 \mu\text{l}$. Cultures were then incubated for 48
20 hours at 37°C and $5\% \text{CO}_2$, and cell proliferation was measured either by direct count in the hemocytometer, or by the MTT assay (Denizot and Lang (1986) *J. Immunol. Meth.* 89: 271-277). Recombinant steel factor (R&D systems, Minneapolis, MN, Cat. No. 455-MC) was used as a positive control, while recombinant CBD was used as the negative control.

25 In experiments where Factor Xa was used to cleave the fusion protein between the SLF and the CBD, the assay was carried out as before except that 2.5 ng of Factor Xa (Boehringer-Mannheim, Indianapolis, IN, Cat. No. 1179 888) were added to test wells during the 12 hour incubation step prior to the addition of the B6SUtA1 cells.

Statistical analysis of data was carried out with regression curve fitting in two
30 iterations using non-linear regression analysis from the GraFit version 3.0 statistical analysis program (Erithacus Software Ltd., Staines, U.K.).

The activity of the non-immobilized SLF-CBD fusion protein as measured by its ability to stimulate proliferation of B6SUtA1 cells was compared to that of the recombinant SLF without an affinity tag (Fig. 6A) using the MTT test and the SLF dependent bone marrow cell line B6SUtA1. The activities of the proteins were
5 similar and within the expected range based on the specific activity of the recombinant SLF. The activity of SLF-CBD was neutralized by anti-SLF neutralizing polyclonal antibodies (Fig. 6B). Recombinant CBD alone did not stimulate the proliferation of B6SUtA1 cells.

10 Biological activity in the presence of BMCC

Bacterial microcrystalline cellulose (BMCC) is a highly crystalline form of cellulose produced by *Acetobacter xylinum* (ATCC 23769). After preparation, BMCC forms a high surface area fine suspension which does not sediment rapidly, and is therefore easily diluted to obtain a desired final concentration. A dilution series
15 of BMCC was prepared, covering a wide range of BMCC concentrations, and each of these concentrations was tested for its ability to stimulate proliferation of B6SUtA1 cells using a constant amount of SLF-CBD. For SLF-CBD at 130 pM, maximum activity was observed with a BMCC concentration of about 1 μ g/ml (Fig. 7A). The activity increased and the peak shifted to the right when the SLF-CBD concentration
20 was increased to 1500 pM (Fig. 7B), indicating that the concentrations of both SLF-CBD and BMCC affect the proliferation response of B6SUtA1 cells in this system and suggesting the need for an optimal surface concentration of SLF-CBD on the BMCC.

25 Influence of SLF-CBD bound to a fixed amount of BMCC

Using a fixed BMCC concentration of 1 μ g/ml or 14 μ g/ml a converse series of experiments was carried out in which the amount of BMCC surface available was held constant and the SLF-CBD concentration was varied. The presence of BMCC had no effect on the activity generated by the non-affinity tagged control SLF over a
30 range of 0 to 2000 pM (Fig. 8A), however SLF-CBD was more active in the presence of BMCC than in the absence of BMCC, with both an increase in total cell

proliferation and a decrease in its ED_{50} (the protein concentration required to produce a half maximal response) being observed (Fig. 8B).

To determine the distribution of SLF-CBD, the activities of free and bound SLF-CBD were tested separately (Figs. 9A-9C). Various amounts of SLF-CBD were bound to 1 $\mu\text{g/ml}$ of BMCC. The matrix was then removed from solution by centrifugation and resuspended in fresh medium. The activities of the original medium and the resuspended BMCC were then assayed for activity to determine how much of the protein bound to the matrix. For the non-tagged SLF control most of the cell proliferation activity was associated with the supernatant (Fig. 9A). The reverse was seen for SLF-CBD, where most of the cell proliferation activity was associated with the BMCC particles (Fig. 9B). This test also demonstrates that the binding capacity of 1 $\mu\text{g/ml}$ of BMCC was not exceeded even by the highest concentrations of SLF-CBD used. SLF-CBD bound to BMCC was released by adding Factor Xa to cleave the fusion protein at the Factor Xa site introduced between the SLF and the CBD. Following this treatment, the bulk of the cell proliferation activity was found in the supernatant (Fig. 9C). These results indicate that, although the maximum cell density generated by the fusion protein in soluble form was not quite as great as that generated by the control protein, the ED_{50} values were identical. When the SLF-CBD fusion protein was immobilized on cellulose, however, it was significantly more potent than SLF-CBD in solution, generating a significantly higher maximum proliferative response and exhibiting a higher specific activity. This effect was not observed for the control protein which lacked an affinity tag and could not be immobilized.

It is noteworthy that the enhanced proliferative activity of immobilized SLF-CBD was dependent on the concentration of BMCC. At a fixed SLF-CBD concentration, the proliferative response increased as the BMCC concentration increased to a maximum value, and then decreased at higher BMCC concentrations. Increasing the concentration of SLF-CBD in this experiment shifted the maximum to higher BMCC concentrations (Fig. 7B). Presumably at very low BMCC concentrations the matrix surface area is limiting while at very high BMCC concentrations it is the steel factor which is limiting, with a decrease in the density of SLF-CBD on the BMCC surface. Increasing the SLF-CBD concentration counteracts

this effect, providing a higher surface concentration of SLF-CBD. The specific surface concentration of steel factor molecules has a dramatic effect on the stimulation of B6SUtA cells. For a SLF-CBD concentration of 130 pM, the addition of BMCC produced a maximum at 1 g/ml, whereas at a SLF-CBD concentration of 1500 pM the maximum shifted to the right to 4.4 µg/ml and the level of stimulation increased (Fig. 7). The level of stimulation in these cultures therefore is influenced both by the surface concentration of SLF and the amount of cellulose surface. Based on the dimensions of the BMCC particles reported by Gilkes *et al, supra.*, the available surface area is 1.22 cm² per µg of BMCC. For both concentrations of BMCC, the density of SLF-CBD on the surface is about 4x10¹⁰ molecules/cm². Assuming a cell diameter of 10 mm and 35,000 *c-kit* receptors per cell as is the case for MO7e cells (Turner *et al.* (1995) *Blood* 85:2052-2058), the receptor density on the cell surface would be 1.1x10¹⁰ receptors per cm². Based on these estimates, the effect of the addition of an immobilization matrix was maximal when the relative density of SLF-CBD was four times the density of the *c-Kit* receptors on the cell membrane.

The effect of increasing SLF-CBD with surface area maintained constant at 1 µg/ml (1.22 cm²/ml) also was analyzed. In this case, maximum stimulatory activity was observed when the SLF-CBD concentration was about 25 pM equivalent to a SLF-CBD density of 1.23x10¹⁰ molecules per cm². This number is about 60 times the estimate we have used for receptor density on the cell surface. Overall, these results suggest that there is a threshold for stimulation when the density of SLF is about four times of the receptor density on the cell surface. The stimulatory activity increases with the SLF concentration reaching its maximum when the SLF surface density is 30-60 fold higher than the receptor density on the cell membrane.

25

Example 4

Persistence of SLF-CBD Biological Activity When Immobilized on Regenerated Cellulose Surfaces

A 1% (w/v) solution of cellulose acetate was prepared by adding one gram of cellulose acetate (Kodak Inc.) to 99 ml of acetic acid. One hundred µl of this solution was added to each well of the standard 96 well tissue culture plates used above, and

30

the acetic acid was allowed to evaporate. One hundred μ l of 50 mM NaOH was then added to each well and left for 20 minutes to regenerate a cellulose surface. The NaOH was then poured out, and the wells rinsed three times with PBS. Three hundred μ l of 70% (v/v) ethanol was then added to each well, the plate lid replaced,
5 and the ethanol allowed to evaporate as a sterilization step.

Activity tests were carried out as previously described except that a cellulose acetate derived surface was used to immobilize SLF/CBD instead of BMCC. Twenty μ l protein test samples were put into cellulose-coated wells. Fifty μ l of H-SFM was then added to the wells, and incubated for 12 h prior to the addition of 100 ml of
10 B6SUtA cells for the standard final volume of 170 μ l. Proliferation activity was measured after 48 h of culture using the MTT test with data points collected in quadruplicate. A control group of cells was added to each of four wells in a cellulose-coated plate and to each of four wells in a non-coated plate. These cells were then counted after a 48 hour incubation to determine if the surface exhibited any toxicity.

15 SLF-CBD was bound to cellulose-coated plates as described above, and B6SUtA cells were added as before. After the standard 48 hour incubation period, the cells were moved to a separate plate without SLF-CBD to determine the proliferative activity by the MTT test. The wells of the old plate were washed with 150 μ l of warm H-SFM. After washing, 170 μ l of fresh B6SUtA test cells were added to each well
20 and the 48 hour incubation period was repeated. The entire procedure was repeated so that the surface was used for cell culture a total of three times. In another experiment, SLF-CBD was bound to a cellulose-coated plate and the H-SFM supernatant from these wells was exchanged every 48 hours without the addition of cells. After the third exchange of supernatant, cells were added to the SLF/CBD-coated surface and
25 the MTT assay was performed after the 48 h incubation. Non-tagged SLF was used as a control. In one set of control experiments, SLF was added to cellulose-coated tissue culture wells as before, the surface was seeded with fresh cells three times, and the proliferation measured. In another series of control experiments the SLF-CBD protein was added to tissue culture plates not coated with cellulose. These surfaces were
30 seeded with cells three times and the activity measured.

SLF-CBD also bound to a reconstituted cellulose surface applied to the bottom of a microtiter plate. This system facilitated the separation of the cells from the cellulose surface so that the surfaces could be reused for several consecutive rounds of cell culture. Analysis of the activity bound to the plate or remaining in the supernatant indicated that essentially all of the SLF-CBD was bound (Fig. 10A). Repeated use of the same surface showed no loss of proliferative activity during the three rounds of cell growth (Fig. 10B). Similar results were obtained from a separate plate, identically treated except that cells were not added until the third cycle (Fig. 10B).

It is apparent that the immobilized steel factor is not being completely consumed by the cells, since SLF-CBD-coated plates could be used repeatedly, but may not be reused indefinitely. In contrast, soluble steel factor is internalized and consumed by cells which is likely a homeostatic mechanism to regulate the duration of stimulation (Miyazawa *et al.* (1995) *Blood* 85: 641-649). Immobilization of SLF on a non-internalizable surface would then prevent down regulation by reducing consumption of the growth factor.

Example 5

Stimulation of Murine and Human Cell Proliferation Using SCF-CBD Immobilized on Regenerated Cellulose Surfaces

Bone marrow colony assay

The purpose of this study was to determine the clonogenic progenitor recovery of primary murine bone marrow cells incubated for 5 days in liquid suspension of primary murine bone marrow cells incubated for 5 days in liquid suspension cultures containing SCF or SCF-CBD with or without cellulose. Bone marrow cells were aspirated from the femurs of 8 week old (C57BL/6J x C3H/HeJ) F1 mice bred and maintained in the animal facility of the British Columbia Cancer Research Center. Cells at a final concentration of 1×10^3 cells ml^{-1} were added to 1 ml culture medium containing SCF or SCF-CBD and with or without $1 \mu\text{g/ml}$ BMCC. Murine IL3 (5ng/ml) was added to parallel test cultures because of its reported synergy with SCF. Cultures were then maintained for 5 days at $37^\circ\text{C}/5\% \text{CO}_2$, the cells counted a

second time, and diluted 1 in 10 in Methocult GF M3434 medium (StemCell Technologies) containing 0.9% methylcellulose and a combination of growth factors to support colony formation (Humphries *et al.*, (1979) *Blood* 53:746-763). One ml of each diluted culture was transferred to each of two 35 mm plates, and incubated for 14 days, after which time the number of colonies was scored according to standard criteria. Total colony counts from each pair of duplicate plates were combined, normalized to 10^4 original cells and plotted as a single bar on the graph (Fig. 11A). Error bars are 95% confidence intervals derived from the Poisson distribution. Plates were photographed after a further 14 days of incubation (Fig. 11B). The cellulose concentration was held constant while the SCF and SCF-CBD concentration was varied from 150 to 10,000 pM. Clonogenic progenitor recovery was dose dependent in all cases. The inclusion of IL-3 had a modest effect on the progenitor output (average increase relative to SCF or SCF-CBD was 45%). Importantly, both SCF-CBD and bound SCF-CBD (Fig. 11C,D) supported the recovery of progenitor numbers at least as effectively as unmodified SCF (Fig. 11A,B) demonstrating that modified SCF retained bioactivity for primary hemopoietic cells. The dose response for progenitor recovery with SCF-CBD bound to cellulose appeared equivalent to SCF-CBD as assessed by total progenitor number. Strikingly, however, cellulose bound SCF-CBD greatly enhanced the recovery of progenitors capable of forming large macroscopic sized colonies compared to progenitors recovered from SCF-CBD or SCF stimulated suspension cultures consistent with bound SCF-CBD having enhanced activity on more primitive precursors (Coulombel *et al.*, (1983) *Blood* 62:291-297).

Experiments with human cell lines MO7e and TF-1 were performed. The half-maximal response (ED_{50}) (Table 7) was calculated from the dose response curves (Fig. 27).

Table 7
Effect of bound and unbound SCF-CBD on cell proliferation

Cell Line	ED ₅₀ ± SEM [pM?]			
	SCF-CBD		SCF	
	no cellulose	with cellulose	No cellulose	with cellulose
B6SutA	455 ± 58	65 ± 9	629 ± 65	807 ± 75
MO7e	178 ± 42	37 ± 24	124 ± 30	308 ± 33
TF-1	160 ± 22	31 ± 16	349 ± 64	311 ± 53

5

In the SCF dependent bone marrow cell line B6SutA, CBD alone did not stimulate the proliferation of B6SutA cells. The presence of cellulose had no significant effect on the activity generated by the non-affinity tagged control SCF (Table 7). However, SCF-CBD in the presence of cellulose was more active in all three cell lines than in
 10 the absence of cellulose as evidenced by the decrease in the protein concentration required to produce a half maximal response (decrease in the value of ED₅₀) (Table 7). IL3 did not affect the size of colonies.

Example 6

15 Effect of Cellulose-Bound SCF-CBD on Clustering and Polarization of Receptors

Cell imaging with laser scanning confocal microscopy

Sheets of cellulose were prepared from the cell walls of the marine alga *Valonia ventricosa*. Sheets dried onto glass coverslips were incubated with either
 20 CBD, SCF (R&D Corp., Minneapolis, MN) or SCF-CBD. After washing, B6SutA cells were cultured for 20 h on the cellulose surfaces. For antibody labeling, cells were fixed with 4% paraformaldehyde, rinsed 3 times and permeabilized with 0.5% Triton X-100. The fixed cells were blocked overnight in 2% bovine serum albumin (BSA) and then labeled with antibodies in 1% BSA. Primary antibodies were as
 25 follows: polyclonal, rabbit anti-Cex CBD; monoclonal, biotin conjugated, rat anti-mouse c-kit receptor (Pharmigen, San Diego, CA); monoclonal, mouse anti-

phosphotyrosine (UBI, Lake Placed, NY). Secondary labels were as follows: Fluorescein isothiocyanate (FITC) conjugated goat, anti-rabbit Ig, FITC conjugated goat, anti-mouse Ig (Sigma, St. Louis, MO) and Streptavidin-Texas Red conjugate (Gibco, Grand Island, NY). Immunofluorescence was imaged using the BioRad
5 MRC600 laser scanning system mounted on a Nikon Axiophot microscope fitted with a 60x1.4 NA objective lens. Fluorescein and Texas Red were imaged independently using standard filter sets.

B6SutA cells adhered to cellulose surfaces presenting bound SCF-CBD, but not to cellulose surfaces pre-treated with either SCF or CBD alone. Therefore,
10 adhesion required both the CBD and SCF domains of the fusion protein. Both SCF-CBD and SCF receptors were concentrated in the areas of contact between the B6SutA cells and the cellulose surface (Fig. 12). This is consistent with the mobility of both SCF-CBD on the cellulose surface and the receptors within the cell membrane. Receptor polarization was still apparent 20 h after initiation of the
15 cultures indicative of a persistent source of localized stimulation. Figure 12 shows the initial event in the stimulation of many cell types is the clustering of receptors following their interaction with cytokines or growth factors. Presentation of the cytokines on stromal cells is particularly effective for triggering these responses, presumably because lateral mobility of the cytokines on the surfaces of the presenting
20 cells allows clustering of receptors on the target cells. However, stromal cells produce a variety of molecules, any or all of which may affect the target cells. The method described here allows facile presentation of a single cytokine in the absence of any confounding effects of unknown factors. The advantage of the method is that the mobility of the CBD fusion protein on the cellulose surface, like the lateral movement
25 of cytokines on stromal cells, allows receptor patching on the target cells.

Furthermore, adsorption of the cytokine-CBD fusion to cellulose ensures its presentation in a uniform orientation. Previous efforts to immobilize cytokines and growth factors on culture surfaces involve covalent attachments (Cuatrecasas, (1969) *Proc. Natl. Acad. Sci. USA* 63:450-457; Ito *et al.*, (1996) *Proc. Natl. Acad. Sci. USA*
30 93:3598-3601; Kuhl and Griffith-Cima, (1996) *Nature Medicine* 2:1022-1027; Horwitz *et al.*, (1993) *Molec. Immun.* 30:1041-1048) which precludes lateral diffusion. When IL-2 was attached to a culture surface covalently, division of CTLL

cells was effectively abolished but could be reinitiated by addition of soluble IL-2 (*Horwitz et al., supra*).

Example 7

5 Cytostructural Characterization of Cellulose Bound SCF-CBD Stimulation

A common feature of SCF stimulation of a cell is the rapid ligand-induced internalization and degradation of the ligand receptor complex (Yee, *et al.* (1993) *J. Biol. Chem.* 268:14189-14201). Receptor is cleared from the cell membrane through
10 internalization in a ligand dose dependent manner with a half life of 30 to 40 minutes (Yee, *supra*). Ligand receptor complexes are typically endocytosed through clatherin-coated pits (Yee, *supra*) reference in Yee (1993)). A recent study with fetal derived mast cells used FACS and immunocytochemical analysis to demonstrate that c-kit was rapidly internalized with SCF and that the reappearance of c-kit on the cell
15 membrane required RNA synthesis (Shimizu, *et al.*, (1996) *J. Immunol.* 156:3443-3449). The rapid patching and internalization of c-kit in response to soluble SCF, suggests that the strong binding of SCF-CBD on crystalline cellulose may be impeding this process and thereby prolonging receptor signalling.

Direct confocal imaging of the internalized receptor-ligand complex has not
20 previously been reported. Therefore, cells stimulated with soluble SCF were first imaged to provide a reference for identifying perturbations caused by SCF-CBD localization to cellulose. To investigate the kinetics of normal soluble SCF-induced receptor patching and internalization, live-cell CLSM imaging was performed. CSLM imaging permits the 3-D spatial localization of the ligand receptor complex at the
25 membrane-cellulose interface.

Fixed-cell imaging of SCF-CBD presented on fibers (BMCC) or sheets (Valonia) was used to localize the SCF-CBD ligand and the c-kit receptor. Immunolocalization with antibody specific for the CBD, SCF and c-kit was used. For these imaging experiments, in order to be able to differentiate the cell from its
30 environment, phalloidin was included as a third stain. Phalloidin is a fungal toxin isolated from *Amanita phalloides* which has a high specific affinity for actin filaments (Knowles and McCullough (1992) *J. Histochem. Cytochem.*) ; see also

<http://www.probes.com/handbook/tables/tab11-1.html> for available phalloxin derivatives together with their binding constants for fluorescently labelled actin). Phalloidin tagged with a fluorophore is thus a convenient method to delineate the actin cell envelope and cytoplasm. Staining with antibodies specific for

- 5 phosphorylated tyrosine was used to probe the activation of SCF-CBD-c-kit complexes at the cellulose interface to resolve the polarization of activated c-kit to the foci of cell contacts with the SCF-CBD cellulose. Further, that cellulose presentation of SCF-CBD served to concentrate the signal making it amenable to direct immunofluorescence imaging.

10

Imaging soluble SCF interaction with cells

- Cells stimulated with soluble SCF were imaged using CLSM to immunolocalize c-kit 20 minutes after stimulation. The dose dependence of subcellular localization also was examined to determine whether c-kit trafficking was affected by
- 15 cytokine dose. Factor-starved MO7e cells were incubated for 20 minutes at several doses of SCF and then quickly fixed in 4% PFM. Fixed cells were probed with YB5.B8 antibody and then stained with FITC-labeled secondary antibody and phalloidin-Texas Red. CLSM was used to image cells so that optical cross sections could be collected through the cell body to spatially localize c-kit. Fig. 13 presents
- 20 representative images obtained for MO7e cells cultured at 100, 25, 6.25, 1.55 or 0 ng/ml rhSCF. The scale bar in the bottom left corner represents 15 μm . Each row shows a series of optical sections ($\delta z \sim 1.0 \mu\text{m}$) through a typical cell. Red staining, used to indicate phalloidin-Texas Red binding to the actin cytoskeleton, shows the actin envelope of the cell membrane and the cytoplasmic compartment of the cell
- 25 volume. The dark area within the cell envelope is the nuclear compartment. Green was used to indicate localization of anti-c-kit receptor antibody (YB5.B8). Yellow patches within the cytoplasm were the result of actin (red) and c-kit (green) colocalization.

- Optical sectioning and image stack volume or surface rendering showed that
- 30 the MO7e cells were approximately 18 μm sphereoids. Optical sectioning revealed that receptors were found in discrete bodies within the cytoplasm of stimulated cells. No cytoplasmic c-kit was observed in unstimulated cells. Further, no significant

YB5.B8 antibody staining was observed on the membranes of cells regardless of stimulation. Thus, the internalized clusters of c-kit likely represented a considerable number of aggregated receptors. In general, the number of endosomes containing c-kit and the absolute fluorescence signal observed for individual endosomes was
5 related to SCF dose, although absolute quantification was difficult because of the c-D nature of the samples and the potential for photobleaching during imaging. This is consistent with FACS results reporting the SCF dose dependent loss of c-kit on cell membranes and internalization of SCF on cultured mast cells (Yee).

An inverted-CLSM with a heated stage was used to culture cells under
10 physiologic conditions for short periods. Hepes buffered RPMI media was used for cell culture and no significant pH change was noted over the time course of imaging experiments. B6SutA cells were cultured in 2.5 cm covered petri dishes fitted to the CLSM stage heater. Cells were added to the dish and cultured for 10 minutes to allow the cells to settle to the bottom of the cultured chamber (adjacent to the objective
15 lens). Cells were motile on the untreated glass bottom of the petri dish surface. Soluble FITC-tagged SCF-CBD was added to cultures (100 ng/ml) and 3-D image sets were collected over time. To minimize the photodamage to cells caused by free radical release from excited fluorophores, confocal imaging was limited to 5 sections at 3 μ m spacing. An image stack was collected every 20 seconds for approximately
20 10 minutes. Presented images are the projections of the middle 3 layers of each image stack. Considerable cell movement occurred during the imaging experiment, making it difficult to track individual cells.

Typical images from the sequence are shown in Fig. 14. PMT gain and black levels were set so that the image field was completely dark prior to the addition of
25 FITC-SCF-CBD. In the first frame (t_0), the slight autofluorescence of the cells is visible. Following addition of FITC-SCF-CBD, the image brightness increased immediately in all areas of the image field not occupied by cells, indicating rapid mixing in the cell culture chamber due to thermally-induced convection. The exclusion of the FITC-SCF-CBD from cells was consistent with the greater than 90%
30 cell viability of the cells as demonstrated by trypan blue exclusion. In the first few minutes following addition of FITC-SCF-CBD, a fluorescent halo developed around each cell indicating a concentration of FITC-tagged molecules on the cell membrane.

Over the next few minutes, fluorescent patches developed on the cell membranes. These patches were likely the result of the concentration of bound FITC-SCF-CBD in receptor aggregates. Receptors appeared to form aggregates prior to internalization. Each cell formed 3 or 4 patches on its cell membrane, consistent with the fixed cell imaging results presented above. Patches were discernible within the cell volume after 10 minutes. In control experiments, CBD_{Cex} tagged with the fluorophore Texas Red was added with the FITC-SCF-CBD. CBD_{Cex}-TXRd was excluded from cells and did not concentrate on the cell envelope over the course of the imaging experiment.

10

Imaging cellulose bound CBDSCF interaction with cells

Fixed-cell CLSM imaging of B6SutA cells incubated with CBD-SCF bound to BMCC or Valonia cellulose was used to obtain structural information on the interaction at the cell-cellulose interface. While there is a lack of surface diffusion of the SCF-CBD on cellulose when adsorbed to cellulose, this does not imply that the SCF is immobilized (in a covalent sense) on the cellulose. However, the impediment of normal receptor aggregation could be effected by the high affinity of the CBD for the cellulose surface. In these experiments, CLSM imaging was used to determine the degree of c-kit patching and internalization in response to SCF-CBD adsorbed to cellulose. Three-color imaging was used to colocalize c-kit and SCF-CBD. Phalloidin was again used to delineate the cell envelope and the cytoplasmic compartment.

BMCC fibers provide micron-scale fibrous microcrystalline cellulose surfaces for the presentation of SCF-CBD to cells. The adsorption of SCF-CBD to these fibers thereby facilitates the establishment of spatially discrete SCF signals.

Immunofluorescent CLSM of cells cultured with SCF-CBD adsorbed to BMCC was used to investigate c-kit polarization to the cell- ECM contact foci and confocal imaging was used to determine the concentration of c-kit on the BMCC fibrils relative to the overall distribution of c-kit within the cell.

B6SutA cells were cultured for 20 minutes with BMCC to which SCF-CBD had been adsorbed ($\Gamma/\Gamma_{\max} < 5\%$) or in control wells containing BMCC and soluble SCF, or only BMCC. Cells were fixed with 4% PFM and then labeled with

biotinylated rat anti-murine c-kit and rabbit anti-murine SCF. Cells were stained with phalloidin-Texas Red and Cy5-Steptavidin and goat anti-rabbit-FITC. Fig. 15 presents a series of projections from CLSM image stacks. Projections were used to flatten the image stack and give an enhanced-focus 2-D representation of the cell. The
5 scale bar in the bottom right panel represents 5 μ m.

Fig. 15A shows a cell stimulated with soluble mSCF and probed with anti-SCF antibody. The formation of round patches on the cell membrane and in the cytoplasm is evident. These patches colocalized with c-kit staining. In contrast, cells stimulated with SCF-CBD on BMCC show fibrous patches consistent with the shape
10 of BMCC fibers when stained with anti-SCF antibody (Fig. 15B). BMCC coated with SCF-CBD adhered to B6Sut-A cells expressing the SCF receptor. Fibers which were treated with CBD alone did not interact with cells. Apparently, the cell is able to interact directly with the fiber presented SCF-CBD causing the fiber to conform to the round shape of the cell body.

15 Cells were counter-stained with anti-c-kit antibody (Fig. 15C). The fibrous appearance of the patches in Fig. 15B is again evident, indicating colocalization of the c-kit receptor with the BMCC presented SCF-CBD. Colocalization was confirmed as shown in Fig. 15D. In this image, the cell has been stained with anti-SCF (green) and anti-c-kit (blue) antibodies and phalloidin-Texas Red (red). Colocalization of the SCF
20 and the c-kit receptor is shown by the addition of green and blue colors which result in the aquamarine fibers shown on the cell surface. The lack of "green" fibers on the cell surface indicates that fibers are essentially coated with c-kit along most of the length of fiber. Some non-SCF-associated c-kit staining was noted in some cells. It is unclear whether this represents newly synthesized c-kit or c-kit internalized without
25 the SCF-CBD which remained associated with the cellulose fiber.

A small aggregate of SCF-CBD-coated BMCC fibers is evident towards the right of panels B and D. The lack of anti-c-kit antibody staining of the noncell-associated fibers demonstrates the specificity of the antibody. No staining was observed in control samples processed without primary antibodies. Further, no
30 BMCC association with cells treated with soluble SCF was found. Cells stimulated with FITC SCF-CBD adsorbed to BMCC fibers appeared the same as the cells in panel B. Contrary to the images presented for soluble factor, fibers do not appear to

be polarized to a specific region of the cell membrane, suggesting an initial homogeneous distribution of receptors.

Valonia cellulose sheets provide centimeter scale planar crystalline cellulose surfaces for the presentation of SCF-CBD to cells. The adsorption of SCF-CBD to these sheets thereby facilitates the establishment of a spatially polarized SCF signal. Immunofluorescent CLSM of cells cultured on Valonia surfaces with bound SCF-CBD therefore permits the direct investigation of c-kit polarization in response to surface presented SCF. The diffusive mobility of receptors in the cell membrane suggests that the cellulose presented SCF-CBD will capture the mobile c-kit when it encounters the SCF ligand. Confocal imaging therefore can be used to determine the concentration of c-kit at the cellulose interface and the distribution of c-kit within the cell membrane.

B6SutA cells were cultured for up to 2 hours on Valonia cellulose sheets mounted on normal glass coverslips. FITC-SCF-CBD were adsorbed in half of the wells ($\Gamma/T_{\max} < 5\%$). Soluble SCF was added in the other half of the wells to give a "spatially homogeneous" growth-factor signal. The cells were fixed with 4% PFM and immunoreacted with biotinylated Rat anti-murine c-kit. Cells were then stained with phalloidin-Texas Red and Streptavidin-Cy5. The cells remained firmly attached to the cellulose during antibody staining. Three-color CLSM was performed: each channel was collected independently, optical sectioning had slice spacing on the order of .15 μm . A 60-degree rotation was then independently projected for each channel using surface rendering algorithms in NIH image. Final images were formed by recombining the independent RGB channels into a single 24-bit, 3-color image.

In Fig. 16, images are presented of a cell cultured on a Valonia surface for 20 minutes. The composite image is from 4 frames taken from a 60-degree rotation projected image stack. The scale bar in the upper left-hand frame represents 10 μm . The frames are at 30, 45, 70 and 90 degrees to the surface normal. The images show the polarization of c-kit (green) at the cellulose interface coated with SCF-CBD (blue). The projections confirm that c-kit antibody bound only under cell bodies (red) and did not appear to bind elsewhere on the cell envelope. This indicates a near complete polarization of c-kit over the 20 minutes of cell exposure to the treated cellulose surface.

The 30 and 45-degree projections show that anti-c-kit antibody staining is concentrated on a portion of its surface contacts. The distribution of c-kit to the "foot-like" structure (arrow: frame A) is consistent with images collected from soluble SCF-stimulated cells except that the c-kit is completely polarized to the basal surface of the structure. The apparent absence of internalized receptors is in direct contrast to images of c-kit internalization collected from solubly-stimulated cells. In control samples stimulated with soluble SCF, no polarization of c-kit receptors was observed, although very few cells remained on the Valonia surface during antibody labeling. Samples prepared without the anti-c-kit primary antibody showed no binding of labeled secondary probes.

Activated receptor was found in the insoluble fraction of the cell lysate. This suggests that to directly image whether c-kit forms a stable complex with the cellulose bound SCF-CBD, cells were cultured on Valonia sheets and then processed as above. Prior to imaging, a subset of the samples were shaken and rinsed vigorously to remove bound cells. Imaging of these samples revealed the apparent c-kit footprint of cells removed from the Valonia surface. Surfaces treated with CBD_{Cex} only did not bind cells or show regions of c-kit stain. This supports the hypothesis that receptors do indeed form stable interactions with cellulose presented SCF-CBD.

As shown above, soluble SCF is rapidly internalized following binding to c-kit, whereas SCF-CBD forms a stable interactions with c-kit. The following experiment was designed to evaluate whether cellulose is internalized with the c-kit ligand complex during normal receptor endocytosis. B6SutA cells were incubated with BMCC with or without SCF-CBD for 20 minutes, fixed, and then stained with rabbit anti-SCF antibody (green), biotinylated rat anti-c-kit (blue) and phalloidin (red). Fig. 17 shows a rotation series of volume rendered confocal image stacks. The frames are at 30-degree intervals rotated about the z axis. Colocalization in this image is represented as the color addition of localized labels (e.g., aquamarine is green/blue and white is green/blue/red).

From the rotation series, it can be seen that some fibers are fully contained within the cell cytoplasm. The arrow in the center image points to a fiber near the center of the cell volume; the fiber remains in the center of the cell body as the image is rotated about its axis. The fibrous morphology of the BMCC fibrils is retained

indicating that the fiber was not condensed at the cell membrane prior to internalization.

Other images show the partial internalization of BMCC fibers suggesting that internalization is initiated at some point along the fiber and the rest of the fiber is carried along. Fibers stained with CBD_{Cex}-FITC did not interact with cells and no nonspecific internalization of fibers was observed. Internalized fibers were also observed in control experiments in which FITC-SCF-CBD was used and cells were not permeabilized prior to imaging.

Three-color imaging next was used to colocalize c-kit and phosphorylated tyrosine residues. The results with fibrous and planar-sheet cellulose morphologies were compared. C-kit internalization and targeting for degradation requires an active tyrosine kinase domain (Yee, *et al.* (1994) *J. Biol. Chem.*, 269:31991; Turner, *et al.* (1995) *Blood*, 85(8):2052), however, it currently is unclear as to whether normal c-kit is recycled back to the cell membrane following ligand dissociation in endosomes.

For experiments with Valonia presented SCF-CBD, cells were cultured for 12 hours under various treatments and then processed as already described for imaging. The monoclonal antibody (4G-10) used in the immunoprecipitation westerns was used for immunolabeling. Cells were counter stained with biotinylated rat anti-c-kit antibody. Goat anti-mouse-FITC and Streptavidin-Cy5 were used as secondary stains. Two-color CLSM imaging was performed with a 60-X lens and confocal aperture yielding a δz of about 0.15 microns. Anti-phosphotyrosine antibodies colocalized with anti-c-kit antibodies in the optical slice at the cellulose surface (Figs. 18A and 18B). Little cell-associated staining was observed away from the interface. This suggests that at least some of the anti-phosphotyrosine antibody labeling was activated due to activation of c-kit. It is however quite likely that other phosphorylated molecules associated with the activated c-kit through SH2 docking sites on the activated receptor. Molecules such as activated PLC-gamma and GAP may contribute to the observed signal.

An optical cross section was performed across the cell at the slice indicated by the arrows in the figure. Images of axial distributions of activated receptors showed that receptors concentrated where the cell's membrane contacted the cellulose surface (Figs. 6, 18C and 18D). Cellulose association of activated c-kit is consistent with the

results from IP westerns. further, the image was collected after 12 hours of culture; length of this receptor activation is considerably longer than has been reported previously although it is unclear as to whether the imaged c-kit is the result of receptor-ligand stabilization over the extended time course of this experiment or is
5 newly-arrived receptor following normal cycling.

Example 8

B6SutA Cells Are Retained by SCF-CBD bound to Gauze

10 Four mL of a liquid mixture containing B6SutA cells prelabeled with "Celltracker Green" cytoplasmic dye (excitation λ /emission λ 485/535, Molecular Probes Inc.), were delivered slowly through a syringe containing packed gauze (1.0 cm²) with or without bound SCF-CBD (10 μ g). Fractions of 0.3 mL were collected from the outflow and analyzed for fluorescence. When 0.5 mL of liquid was
15 left in the syringe, 0.5 mL of a Trypsin/EDTA solution (Gibco BRL) was pulled into the syringe and the sample incubated at 37°C for 10 minutes to release cells adhered to the gauze. The remaining liquid was then delivered from the syringe and collected as before. An increase of fluorescence was observed in the fraction collected after trypsin treatment, the gauze presented SCF-CBD (arrow) (Fig. 19). This result
20 indicates that SCF-CBD can mediate cell retention to a cellulose matrix. The open circles represent the gauze without bound SCF-CBD; the closed circles represent the gauze with bound SCF-CBD.

Example 9

25 Comparison of SCF-CBD Attachment to BMCC, CELLEX MX and Avicel

After mixing 1.0 μ M SCF-CBD solution with a test cellulose matrix at various concentrations for 12 h at 4°C, B6SutA cells were added to a final concentration of 2×10^5 cells/mL. After 48 hours of incubation, cell stimulation was estimated with the
30 MTT assay. Addition of CELLEX MX (Biorad) enhanced the potency of the cytokine over a wider range of concentrations than the other matrices tested. Avicel (FMC) also showed an appreciable effect. A control sample with no matrix was included in each

test (black bars). These results indicate that both CELEX and Avicel can be used in cell culture to enhance the activity of SCF-CBD. The results are shown in Fig. 20.

Example 10

5 Binding of CBD-Fusion Proteins to Polymers

Preparation of EHEC-coated plates

Three types of EHEC were obtained from Akzo-Nobel Sweden. The three samples differ in their degree of polymerization and subsequently in their molecular
10 weight but have a similar degree of substitution.

Bermocoll E 230 FQ lot # 12035

Bermocoll 351 X lot # 02095

Bermocoll E 481 FQ lot # 01677

1 mg EHEC/ml PBS (Sodium Phosphate buffer saline pH 7) was prepared as
15 follows: 500 mg of EHEC was suspended using moderate stirring in 100 ml of double-distilled water (DDW) at 80°C after which ice-cold 350 ml DDW were added and the solution was kept stirred until the EHEC was fully dissolved, then 50 ml of 10xPBS were added.

Polystyrene microtiter plates were soaked overnight in the EHEC solution at
20 room temperature (RT) making sure that no air bubbles were trapped in the wells. The next day, the plates were rinsed with DDW and either air dried at RT overnight or for several hours at 60°C.

25 Binding of proteins to EHEC-coated vs. polystyrene and polypropylene plates and determine bound proteins by micro-BCA assay

Protein solutions 100 µg/ml PBS were prepared and their concentrations were determined by micro-BCA assay (Pierce) in microtiter plates using 100 µl of protein solution plus 100 µl of the reagent. The plates were covered with Saranwrap, placed at 60°C for 1 h, cooled to RT and the OD was determined at 562 nm. Albumin was
30 used as a standard.

Streptavidin, CBDclos-Streptavidin, GFP and CBDclos-GFP were used. Aliquots of 100 µl were added to the wells in triplicates. The plates were incubated at

RT for 2 h or in some experiments overnight at 4°C then washed 4 times using 200 ul PBS.

Bound protein was determined as follows: 100 µl of PBS plus 100 µl of micro-BCA reagent were added to each well and the plates were covered with Saranwrap, placed at 60°C for 1 h, cooled to RT and then the OD was determined at 562 nm.

Determination of biotin binding CBD-Streptavidin and Streptavidin-coated plates

Plates were incubated with 100 µl of 100 µg/ml biotinylated-HRP (Pierce cat. # 29319) for 1 h, then washed 4 times with PBS containing 1.0% of Tween-20. Aliquots of 100 µl HRP substrate at RT (2,2'-Axino-bis-(3-ethyl-benzthiazolin-6 sulfonic acid), ABST reagent Calbiochem cat. # 194434) were added and the reaction was stopped after 30 seconds by adding 100 µl of 0.5M H₂SO₄. OD was determined by ELISA reader at 405 nm within 30 minutes.

The binding of different proteins and their CBD-fusions to EHEC-coated plates vs. polystyrene and polypropylene is shown in Fig. 21. The EHEC polystyrene and polypropylene bind CBD fusion proteins better than the same proteins without the CBD moiety. Streptavidin binds to polystyrene but very little to EHEC or polypropylene. These results indicate that CBD facilitates the binding of proteins to different polymers, most likely via the hydrophobic amino acids located on its cellulose binding face.

The net signal intensity of CBD-Streptavidin was better than that of Streptavidin in all three polymers (Fig. 24). Streptavidin did not bind significantly to EHEC-coated plates as shown in Fig. 25, which explains the low HRP signal that resulted from lack of biotin binding.

Example 11

Surface Diffusion of Cellulases and Their Isolated Binding Domains on Cellulose Protein production and purification

30

The genes encoding the exoglycanase Cex or the isolated CBD_{Cex} were subcloned into the pTZE07 vector and expressed in *E. Coli* JM101 (Ong *et al*

- Biochemistry* (1993) 42:401-409). The gene fragments encoding the catalytically inactive mutant of the endoglucanase CenA (Asp252Ala) and the isolated CBD_{CenA} were subcloned into the vector pUC18 and expressed in *E. Coli* JM101 (Damunde *et al Biochemistry* (1995) 34:2220-2224). Fermentations were carried out in a 20-L Chemap fermenter at 37°C. Cellulases and their isolated CBDs were purified by affinity chromatography on Avicel PH101 (FMC; Ireland, County Cork), a microcrystalline form of cellulose (Gilkes *et al J. Biol. Chem* (1992) 267:6743-6749. Contaminating oligosaccharides from the Avicel affinity column were removed by size exclusion chromatography on a Superose-12 column (Pharmacia: Uppsula, Sweden) (Creagh *et al Proc. Natl. Acad. Sci USA* (1996) 93:12229-12234.

Protein labeling with Fluorescein

- CBD_{Cex} has only two amino groups which can react with fluorescein isothiocyanate (FITC): the N-terminus, and a single, surface-exposed lysine residue.
- Neither is on or near the putative binding face of the CBD (Xu *et al. Biochem.* (1996) 34:6993-7009. CBD_{CenA} also has 2 potential reaction sites which are sufficiently removed from the binding face so that FITC-labeling does not influence adsorption characteristics. Proteins were labeled by standard procedures (Brinkley, M. *Perspectives in Bioconjugate Chemistry* (1993) (ed. Meares, CF.ACS.) Washington 59-70). Briefly, 0.15 mg FITC was added per mg of protein at 1 mg protein per ml. The pH was adjusted to approximately 9 to initiate the reaction and the solution was gently mixed in the dark at 4°C for 5 hours. The labeled protein was passed twice through a 5 ml Sephadex G50 column (Pharmacia) in a 50 mM phosphate buffer mobile phase to separate unbound FITC from labeled protein. Fractions containing significant amounts of protein were pooled and the absorbance at 280 nm and 495 nm was used to determine the number of FITC molecules bound per protein. On average, 1.5 moles FITC bound per mole of CBD protein. A slightly higher conjugate ratio of *ca.* 2.2 was found for whole enzymes. Protein solutions were stored in the dark at 4°C until use.

Valonia ventricosa microcrystalline cellulose

Valonia ventricosa is a marine algae which grows in many temperate marine environments. Its cell wall has a multilamellar structure organized with each lamella positioned orthogonal to its neighbor. Each lamella contains several parallel layers of cellulose microfibrils (Revol, J.F. *Carbohy. Poly.* (1982) 2:123-134). The individual microfibrils are highly crystalline with a square cross section of approximately 18 nm corresponding to the 220 and the $\bar{2}\bar{2}0$ crystallographic planes. Electron diffraction measurements show that the $\bar{2}\bar{2}0$ face of the microfibrils is preferentially oriented parallel to the cell wall. Both orientations of the microfibril longitudinal axis occur within each lamella.

Preparation of cellulose surfaces for analysis

Preparation and cleaning of *V. ventricosa* cell walls was based on the method of Gardner and Blackwell (Gardner *et al Biopoly* (1974) 13:1975-2001). For FRAP analysis, cell wall layers were carefully peeled apart under a dissecting microscope, typically into about six distinct sheets. The outermost and innermost sheets were discarded. Each of the remaining sheets were then floated and spread evenly onto a normal No. 1 glass coverslip (Baxter Canlab; Montreal, Canada). After drying, the sheet was trimmed to a 3.5 mm square and permanently fixed to the coverslip using a narrow border of Quickmount mountant (Fisher Scientific; Vancouver, Canada). Microscopic examination showed that the mountant did not permeate past the perimeter of the cellulose sheet. Mounted cellulose samples were stored at room temperature.

Binding of proteins to *V. ventricosa* cellulose sheets

A short length of tubing was fixed to the dried cellulose sheet on the coverslip to form a well. Prior to binding CBDs, the well was filled with 50-mM phosphate buffer, incubated for 10 minutes, and then inverted and gently shaken to remove excess liquid. 400 μ l aliquots of labeled protein diluted in 50-mM phosphate buffer were then added to the wells. Equilibrium between the bound and free protein fractions was reached within 3 hours, after which the supernatant was removed and set aside for subsequent determination of the unbound protein concentration. The sheet

was rinsed thoroughly with 50-mM phosphate buffer and then soaked for 30 minutes with a buffer change after 15 minutes. The tube which formed the well over the cellulose sheet was removed and the cellulose sheet was mounted over a small well drilled into a normal microscope slide (baxter) (approximate volume 8 mm³) containing 50-mM phosphate buffer. The coverslip was sealed around the well with silicon grease to prevent evaporation during imaging.

For comparison with previous results, CBD_{Cex} binding isotherms were determined using *V. ventricosa* cellulose from sheets disrupted by sonication. A new approach to CBD binding analysis was developed to permit analysis of protein concentrations at pmole levels. Briefly, disrupted cellulose and FITC-labeled CBDs were added to an eppendorff tube which had been "preblocked" with bovine serum albumin to minimize nonspecific adsorption to the container walls. The filled tubes were placed on rotating mixers for 3 hours at 25°C in the dark to allow binding to come to equilibrium. Following binding the tubes were centrifuged at 10,000 RPM for 10 minutes to pellet the cellulose adsorbent. The supernatant was collected and added to preblocked [?] eppendorff tubes containing a 25-fold excess of Avicel (based on saturation capacity) to concentrate the unbound CBD-FITC. Samples containing a known amount of CBD-FITC (standards) were prepared in a similar fashion. Tubes were then placed on a rotating mixer for 24 hours at 25°C in the dark. Following binding, the tubes were spun at 10,000 RPM for 10 minutes to pellet the cellulose adsorbent. The Avicel pellet was resuspended in 100 µl of 50-mM phosphate buffer and transferred to wells in a 96-well plate. Sample fluorescence was determined using a 96-well plate fluorimeter (IDEXX; Portland, MA) at an excitation wavelength of 488 nm and emission wavelength of 535 nm.

Figure 19A shows images of a mounted cellulose sheet stained with the fluorescein labeled binding domain of exoglycanase (Ces(DBD_{Cex}-FITC). The parallel-array microfibril structure and uniformity of the surface is evident. *V. ventricosa* cellulose has a high degree of crystallinity (> 95% Gardner *et al Biopoly* (1974) 13:1975-2001), and a high binding capacity for CBDs. Uniform, flat regions on the cellulose surface were selected for photobleaching experiments. Figure 19B shows a cross section perpendicular to the surface at the axis indicated in Figure 19A. The sheets of *V. ventricosa* cell wall prepared for our studies are approximately 1 µm

thick. The axial resolution of the confocal microscope under our imaging conditions is on the order of 1 μm . All fluorescence signals collected during recovery monitoring thus arise within or near the cellulose sheet.

To ensure that binding of labeled and unlabeled CBDs was equivalent, binding isotherms were measured for mixtures containing 10%, 20% and 50% labeled CBD_{Cex}. The affinity constants and saturation capacities of the CBD on cellulose for the mixtures were in quantitative agreement, indicating that FITC labeling of the CBD did not affect its binding properties. Quantitative fluorescence microscopy and isotherm analysis showed that CBD-FITC fluorescence intensity per mole of bound protein was independent of surface concentration. Thus FITC self-quenching at high surface concentrations is not a concern.

FRAP Analysis Using A Confocal Microscope

The BioRad MRC 600 confocal microscope (BioRad, Richmond, CA) used for imaging and FRAP experiments consists of laser scanning mirrors, filters for excitation and emission, and photomultiplier tube(s) (PMT) mounted onto a conventional Nikon Optiphot-II microscope. 10 X (N.A. 0.8) and a 60 X (N.A. 1.4) objective lenses were used for imaging. A 100-mW Kr/Ar laser was used for excitation at 488 nm. Excited fluorescence intensity was measured using a 535 nm bandpass filter and PMT. The PMT gain was adjusted to maximize the dynamic range in all images. The PMT black level was set at 4.7 for all imaging. The confocal aperture is noted each image in the figure legends.

FRAP is typically performed using a laser spot focused through a microscope on the surface to be investigated. The laser is equipped with a shutter which permits the rapid attenuation of beam intensity so that recovery can be monitored following bleaching. Confocal laser scanning microscopes (CLSM) have recently been used for FRAP analysis (e.g. Blonk *et al J. Microscopy* (1993) 169:363-374; Peters *et al Biochim. Biophys. Acta* (1974) 367:282-294). CLSM has the significant advantage of permitting recovery monitoring at a defined image plane of the specimen. For relatively slow transport processes, a CLSM without a rapid laser attenuation shutter can be used for FRAP analysis. Slower recovery processes also permit the acquisition of entire image planes which may include several bleached regions on the sample. In

principle, this permits the determination of diffusive anisotropy across the surface under investigation.

For FRAP analysis, a 0.06% transmission filter was placed in the laser path in front of the instrument's standard filter set to attenuate the laser for recovery monitoring. The neutral density filter wheel on the BioRad instrument was set to 3 (3% transmission) during all imaging scans. An image collected prior to bleaching was used to normalize fluorescence intensities to prebleach levels. The CLSM was then electronically zoomed (zoom = 8) so that only a small region of the surface was illuminated during laser scanning. One scan was performed at this high zoom to produce a large, bleached reference region. The CLSM zoom was then returned to its normal setting (zoom = 2) and the neutral density filter wheel on the BioRad instrument set to 0 (100% transmission). Using six successive laser parking and shutter opening (~100 msec each) sequences, six bleached spots were produced for FRAP analysis (Figure 21). The neutral density filter was then returned to the 3 position and recovery monitoring initiated. Fluorescence intensity was monitored until greater than 95% of fluorescence recovery had occurred.

The center of each bleach spot was determined by averaging several successive bleach spot images and selecting the pixel with the minimum intensity as the bleach spot center. The bleach spot intensity profile was then radially averaged to obtain an intensity cross section with spatial heterogeneity of the crystalline cellulose microfibrils averaged out. A gaussian curve was fit by nonlinear least squares regression to the averaged radial profile of fluorescence intensity within the bleach spot. In all cases the averaged spot profile was well fit by a Gaussian function. The parameters from the Gaussian fit (width, depth and offset) were used to calculate the fluorescence intensity at the spot center. The offset value (fluorescence intensity three spot diameters from the spot center) was used to estimate the "background" fluorescence bleaching occurring during recovery monitoring. Laser intensity was attenuated to minimize bleaching during recovery monitoring (less than 5%). Smoothed recovery time-profiles were normalized against the initial prebleach fluorescence. The surface diffusion coefficient and mobile fraction were determined from the normalized recovery curves by nonlinear regression of the parameters in the

series solution for spot FRAP diffusion analysis developed by Axelrod (Alexrod *et al Biophys. J.* (1976) 16:1055-1069.

Figure 26 shows the binding isotherm for FITC-labeled CBD_{Cex} on disrupted cellulose fibers. The use of fluorescently labeled protein permitted data to be collected at much lower concentrations than have been reported previously. The adsorption isotherm data was analyzed by nonlinear regression using a model which includes two classes of binding sites (Creagh *et al Proc. Natl. Acad.Sci.* (1996) 93:1229-1234). Table I reports regressed binding constants and capacities. The saturation capacity of *V. ventricosa* cellulose was determined independently using a mixture of labeled and unlabelled protein. Thus, three parameters were regressed from the adsorption isotherm: high and low affinity constants and fraction of high affinity sites. The binding parameters of CBD_{Cex} agree well with earlier values determined using unlabelled CBD and bacterial microcrystalline cellulose derived from *Acetobacter xylinum* (Creagh *et al Proc. Natl. Acad.Sci.* (1996) 93:1229-1234).

15

Table I
Absorption Isotherm Analysis for CBD_{Cex} on
Crystalline Cellulose

<u>Sorbent</u>	<u>Association Affinity Constant</u>	<u>Capacity</u>
	(μM^{-1})	($\mu\text{mol/g}$)
V. ventricosa cellulose	K ₁ 50 x 10 ⁶	5.0
	K ₂ 1.0 x 10 ⁶	1.2
BMCC	K ₁ 63 x 10 ⁶	3.43
	K ₂ 1/1 (± 0.6) x 10 ⁶	0.9 (± 0.5)

20

Previous studies indicate that the binding of CBD_{Cex} to bacterial microcrystalline cellulose (BMCC) is irreversible (Creagh *et al Proc. Natl. Acad.Sci.* (1996) 93:1229-1234). The binding characteristics of CBD_{Cex} to BMCC and *V. ventricosa* cellulose are similar (Table I), consistent with the highly crystalline nature of these two substrates. However, it was not certain that the binding of CBD_{Cex} to *V. Ventricosa* cellulose was also irreversible. Because irreversible association is a critical

25

condition in our interpretation of the FRAP measurements this question was examined in detail. First, FITC-labeled CBD was adsorbed to cellulose sheets according to the protocol for FRAP analysis. Following washing, the protein-loaded cellulose was equilibrated with 50-mM phosphate buffer. No fluorescence could be detected in the
5 equilibrated buffer solution after 8 hours of incubation, indicating that no CBD had been released from the surface. To demonstrate that CBD was not released from the cellulose surface during FRAP, the bottom side of the microscope slide well was sealed with a coverslip bearing a second unlabelled sheet of *V. ventricosa* cellulose. This surface acted as the capture surface for any CBD which might desorb from the
10 target cellulose surface during FRAP. After a series of FRAP measurements, the top sheet with bound protein, and the bottom sheet which initially had no protein adsorbed to it, were imaged using the confocal microscope. The confocal aperture was adjusted for a depth of field of approximately 1 μm so that fluorescence from opposing walls was excluded. The fluorescence intensity of the unlabelled cellulose sheets did not
15 increase during FRAP experiments confirming that the adsorption of these CBDs to crystalline cellulose is essentially irreversible under these conditions.

Photobleaching analysis of CBD-FITC on crystalline cellulose

Figure 21 shows typical images recorded for FRAP analysis of FITC-labeled
20 CBD_{Cex} on *V. ventricosa* cellulose. Fluorescence intensity measurements of the surface just prior to bleaching (Figure 21A) were used to normalize subsequent measurements to prebleach intensities. Gaussian-profile spots were bleached with a series of high intensity laser pulses in the pattern shown (Figure 21B) and the fluorescence intensity recorded over time by successive imaging of the bleached spots and surrounding area.
25 Approximately seven minutes after bleaching substantial fluorescence recovery is evident in the bleached spots and at the interface between the bleached reference region and the surrounding unbleached area (Figure 21C).

Figure 22A shows the time profiles for fluorescence intensity of the center of the bleach spot, the unbleached region of the cellulose sheet, and the center of the
30 bleached reference region. Under the monitoring conditions selected, background bleaching was less than 5% during recovery. The bleach spot recovery profiles were therefore analyzed directly without compensating for bleaching as a result of

monitoring. Fluorescence did not recover in the large bleached reference region indicating that no highly diffusive species were present on the surface or in solution and that chemical recovery of the bleached FITC was not occurring (Stout *et al Photochem. PhotoBiol.* (1995) 62:239-244).

- 5 Surface diffusion coefficients and mobile fractions were determined by nonlinear regression to FRAP data of the analytical solution developed by Axelrod (Axelrod *et al Biophys* (1976) 16:1055-1069) for the 1-D radial diffusion equation applied to a bleach spot with an initially Gaussian intensity profile.

$$10 \quad f(t) = \sum_{n=0}^{\infty} \frac{(-k)^n}{n!} \frac{1}{1+n \left[1 + \left(\frac{2t}{\tau_d} \right) \right]}$$

- where $f(t)$ is the normalized fluorescence intensity at the bleach spot center, t is time, τ_d is the characteristic diffusion time for the molecular species, and k , the bleach rate
 15 constant, is related to the sensitivity of the system to bleaching. Normalized fluorescence intensities were calculated from raw FRAP data according to

$$f(t) = \frac{[F_k(t) - F_k(0)]}{[F_k(\infty) - F_k(0)]}$$

- 20 where $F_k(t)$ is the measured fluorescence intensity over time, $F_k(0)$ is the fluorescence just prior to bleaching, and $F_k(\infty)$ is the final fluorescence recovered. The first twenty terms in the series given in equation (1), which were more than sufficient for series convergence, were used to regress τ_d and k to normalized FRAP recovery curves such as that shown in Figure 22B. Several initial guesses were used for each fitting to
 25 ensure unique convergence of parameters during fitting.

The diffusion coefficient is related to the estimated characteristic diffusion time by

$$30 \quad D = \frac{\omega^2}{4\tau_d}$$

where w , the half width of the Gaussian profile (at e^{-2} times the spot profiles depth), was obtained by regression of the initial bleach spot profile. The mobile fraction was then determined from the long time recovery intensity by

$$R = \frac{[F_k(\infty) - F_k(0)]}{[F_k(i) - F_k(0)]}$$

where $F(\infty)$ is the effective normalized infinite-time recovery, $F(o)$ the normalized fluorescence just after bleaching, $F(i)$ the normalized fluorescence intensity prior to bleaching and $F_k(i)$ the fluorescence intensity just after bleaching.

Figure 4b shows normalized FRAP results for CBD_{Cex} at 60% maximal surface coverage. Under these conditions, the 2-D diffusion coefficient for CBD_{Cex} on crystalline cellulose is $6.0 \pm .5 (10^{-11}) \text{ cm}^2/\text{sec}$. This diffusion coefficient is more than 4 orders of magnitude slower than the free solution diffusion rate of $10^{-6} \text{ cm}^2/\text{sec}$ estimated from the Einstein equation for a globular protein with a mean diameter of 3 nm. If diffusion is strictly stochastic (i.e. no preferred direction or orientation) this diffusion rate corresponds to a cellobiose unit-cell transit time of approximately of 0.18 msec.

Based on the maximum recovery of fluorescence, the mobile fraction of CBD_{Cex} on the crystalline cellulose surface is $65\% \pm 5\%$. Therefore, on the time scale of these experiments, the majority of the CBD adsorbed to the lattice surface is mobile. FRAP analysis repeated on cellulose sheets stored in buffer at 4°C for 48 hours yielded similar diffusion parameter estimates. There were no obvious changes in the morphology of the cellulose surface or microfibril packaging with incubation time, indicating that the CBD had not significantly altered the cellulose structure.

Surface diffusion rate as a function of bound CBD surface coverage density

Figure 23 shows diffusion coefficients and mobile fractions regressed from FRAP measurements of CBD_{Cex} on *V. ventricosa* cellulose at various fractions of the maximal surface coverage ($\Gamma/\Gamma_{\text{max}}$). Measured binding isotherms for CBDs on the prepared sheets were used to estimate fractional surface coverage densities. The maximal protein loading was 0.4 nmole CBD_{Cex} per cm^2 cellulose. The average sheet thickness was approximately 1.0 μm as determined by imaging several cross sections

using a confocal microscope. Each sheet therefore represents a total cellulose volume of approximately $1.2 \times 10^{-5} \text{ cm}^3$ of cellulose, or approximately $147 \mu\text{g}$ of cellulose per cm^2 of cellulose sheet based on a crystalline cellulose density of 1.5 g/cm^3 . These results give a binding capacity of $5.5 \mu\text{mol}$ CBD per gram of valonia cellulose. This capacity agrees well with values from isotherms prepared using disrupted cellulose sheets, indicating that most of the surface of the undisrupted sheet is available for binding.

The diffusion rate of CBD_{Cex} increases with surface coverage up to a $\Gamma/\Gamma_{\text{max}}$ of ~ 0.9 , after which the estimated diffusion rate decreases as the surface becomes saturated (Figure 23A). At low surface coverages the diffusion rate is about $3.0 \times 10^{-11} \text{ cm}^2/\text{sec}$ increasing to a maximum of about $1.2 \times 10^{-10} \text{ cm}^2/\text{sec}$ at $\Gamma/\Gamma_{\text{max}}$ of ~ 0.9 . The mobile fraction of CBD_{Cex} also increases slightly as a function of $\Gamma/\Gamma_{\text{max}}$ (Figure 23B). At low surface coverages the mobile fraction is approximately 60%. At high surface coverage density, the mobile fraction reaches a maximum of about 85%.

15 Surface diffusion rates for different cellulose binding proteins

Table II presents recovery results for two different *Cellulomonas fimi* cellulases and their respective CBDs at 60% surface coverage. The exoglucanase Cex has little activity on crystalline cellulose (Gilkes *et al J. Biol. Chem* (1992) 267:6743-6749); hence, enzymatic modification of the cellulose surface during the course of diffusion experiments was not a concern. The endoglucanase CenA is moderately active on crystalline cellulose. A catalytically inactive mutant of CenA was therefore used to prevent surface degradation (Damude *et al Biochemistry* (1995) 34:2220-2224). This mutant binds substrate with wild-type affinity but is unable to cleave substrate because the acid catalyst Asp252 is mutated to Alanine. Diffusion coefficients and mobile fractions are presented for equivalent molar concentrations of protein. In both cases the whole enzyme has a significantly higher diffusion rate than the isolated binding domain, with Cex having a higher diffusion rate than CenA. The mobile fraction of CenA is about 85% compared to 70% for Cex. The mobile fractions appear to be a function of the CBD domain and do not depend upon whether the domain is isolated or part of the enzyme.

Table II
Surface Diffusion Rates for Two Different
CBD Containing Enzymes

5

<u>Molecule</u>	<u>Diffusion Coefficient</u>	<u>Mobile Fraction</u>
CenA	$2.9 \pm .5 (10^{-11})$	$0.85 \pm .07$
CenA CBD	$1.9 \pm .4 (10^{-11})$	$0.89 \pm .07$
Cex	$4.1 \pm .5 (10^{-11})$	$0.65 \pm .05$
CexCBD	$3.1 \pm .4 (10^{-11})$	$0.65 \pm .06$

Example 12

Use of Cytokine-CBD as a Gene Transfer Vehicle

10

As we have demonstrated, micron-scale fibrils of crystalline cellulose (BMCC) can be internalized by cells bearing receptors for the growth factor conjugated to the CBD. This suggests that the BMCC fibril may be used as a vehicle for transport of materials (e.g. DNA or anti-sense RNA) into the cell. Furthermore, because the internalization is receptor mediated, only those cells presenting the specific receptor will take up the loaded BMCC fibril. Thus, intracellular delivery of target compounds can be made cell-population specific. For example, in gene therapy applications, a ligand receptor pair could be selected for delivery into stem cells so that the transferred gene might be stably integrated in the host.

Receptor mediated internalization and transformation of B6SutA cells with DNA bound to cellulose (BMCC) carrying SCF-CBD, is demonstrated as an example of the use of cytokine-CBD fusion proteins as a vehicle for transforming cells which possess the receptor for the specific cytokine or growth factor comprising the CBD fusion protein, which is SCF in this case. Sensitivity of B6SutA cells to G418 is determined (Fig. 28) because the vector (pEGFP-N1 vector from CLONTECH) encodes for G418 resistance, in addition to GFP.

Results: 1 mg G418/mL is sufficient to reduce viability to less than 2 % in 48 hours in the conditions tested: 2×10^5 cells/mL in maintenance medium: Iscove's modified Dubelcco's medium containing 10% FCS and 5% HemoStim™ M2100 (Fig. 28). At 0.5 mg/mL, all cells in the culture were non-viable after 120 hours.

5

Adsorption of DNA, and binding of CBD to BMCC

The composition of the samples tested is indicated in the following table:

Sample	BMCC	DNA	CBD
A	+	-	-
B	+	-	+
C	+	+	-
D	+	+	+

10

The above results demonstrate the construction and purification of a growth-factor conjugate that consists of a steel factor extracellular domain and a cellulase polysaccharide binding peptide (SLF-CBD). The SLF-CBD, when bound diffusively to microcrystalline cellulose, stimulates differentiation of steel factor responsive cells to a greater level than non-immobilized steel factor; the immobilized SLF-CBD has both a higher specific activity and a greater maximum response compared to non-immobilized steel factor. SLF-CBD bound to a cellulose surface can be re-used several times without loss of differentiation-stimulating activity. SLF-CBD bound by a cleavable linker and/or at low concentration stimulates proliferation of growth factor dependent cells.

20

A brief stepwise outline of sample preparation is as follows: (1) BMCC + DNA in PBS; (2) lyophilization; (3) resuspension in H₂O/PBS; (4) add CBD/ SCF-CBD; (5) 2 hrs 4°C; (5) fractionation in 2 tubes; (6) centrifugation; and (7) analysis of CBD and DNA in the pellet and supernatant.

25

BMCC (0.1 mg) and DNA (pEGFP-N1 vector, 2 µg) are mixed in 250 µL

PBS and lyophilized in sterile ampoules. After lyophilization, the samples are resuspended by addition of 250 μ L of H₂O. CBDcex or SCF-CBD (0.5 μ g) and PBS (for a final volume of 500 μ L) are added. Following an incubation of 2 hours at 4°C, the samples are spun and resuspended in Iscove's medium (500ul).

- 5 B6SutA cells are incubated in SFM (Iscove's modified Dubelcco's medium supplemented with BSA, transferrin and insulin) for 7 hrs. These factor-deprived cells are then used (5×10^5 cells/well) to demonstrate gene transfer using the following experimental design.

	BMCC	DNA	SCF-CBD	Lypoplectamine
E	+	-	-	-
F	+	-	+	-
G	+	+	-	-
H	+	+	+	-
I	+	+	-	-
J	-	+	+	-
K	-	-	-	-
L	-	-	+	-
M	-	+	-	+
N	-	-	-	+

10

- After an incubation for 3 hours at 37°C, FCS (to 10 %) and Hemostim (to 5%, StemCell Tech. Vancouver, BC)) is added. After an overnight incubation, the cells are resuspended in fresh medium. 48 hrs after treatment, the cells are analyzed using a fluorescence microscope (Bob Harris' lab) using a 480 filter and a 40x immersion
 15 lens. Fluorescent cells are indicative of transfer of the GFP gene. Positive clones can also be detected by FACS analysis. To isolate clones G418 is added to the cultures to kill non-transformed cells. Maintenance medium is replaced twice per week. Clones appear after about 7 days.

- Alternative methods for binding DNA to cellulose, preferably to BMCC,
 20 include: binding DNA to BMCC by conjugation with poly-Lysine, using biotin-labeled DNA (label linearized vector) to bind DNA to BMCC via Streptavidin-CBD, and binding DNA to DEAE-cellulose.

Example 13Preparation of CBD-IL2

5 The construction of the CBD-IL2 expression vector was described by Ong (Ong *et al.*, 1995). Briefly, a plasmid containing the gD1 herpes simplex leader peptide, the P/T linker domain, and the gene fragment encoding CBDCenA were ligated into the pSVL plasmid (Pharmacia; Uppsula, Sweden) to give the plasmid pSVL-gD1-CBDCenA-PT-IL2. COS cells were transfected with the vector and
10 grown in DMEM supplemented with 10% FCS (fetal calf serum) (Gibco BRL), 100 units penicillin ml⁻¹ and 100 mg streptomycin ml⁻¹. Cells were cultured to late exponential phase and then the supernatant harvested by centrifugation at 1500 g. The supernatant was stored at -20° until use. Cells produced between 150-600 mg CBD-IL2 per litre of culture supernatant.

15 IL-2 bioactivity is concentrated onto cellulose, directly from tissue culture supernatants from transient transfections of COS cells with the CBD-IL2 construct. The binding capacity of Avicel is estimated to be at least 500 Units/mg. Native IL-2 does not bind to Avicel cellulose (Greenwood, 1993). CBD-IL2 loaded cellulose particles are then used for culture of IL-2 dependent cells including T-cells and NK
20 cells. Stimulated cells may then be recovered from the culture and reinfused into the patient for immune therapy.

 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference
25 to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

 The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A composition comprising:
a complex comprising a viable cell containing a biologically active growth factor receptor and a ligand for said receptor immobilized on a biocompatible solid
5 phase via a polysaccharide binding domain, wherein said polysaccharide binding domain diffuses on said biocompatible solid phase, and wherein said cell surface localized growth factor receptor diffuses within said cell surface.
2. The composition according to Claim 1, wherein said biocompatible solid
10 comprises a water insoluble cellulosic.
3. The composition according to Claim 1, wherein said biocompatible solid comprises a solid support coated with a biocompatible polymer.
4. The composition according to Claim 2, wherein said water insoluble cellulosic
15 is selected from the group consisting of amorphous cellulose, semi-crystalline cellulose and crystalline cellulose.
5. The composition according to Claim 3, wherein said water biocompatible polymer is ethyl hydroxyethylcellulose or methylhydroxyethylcellulose.
20
6. The composition according to Claim 1, wherein said polysaccharide binding domain comprises a cellulose binding domain.
7. The composition according to Claim 1, wherein said ligand comprises a stem
25 cell factor.
8. The composition according to Claim 1, wherein said biocompatible solid phase comprises a beta-1,4-glycan.
- 30 9. The composition according to Claim 8, wherein said beta-1,4 glycan is chitin or a chitin derivative.

10. The composition according to Claim 1, wherein said ligand is a small molecule mimetic of a growth factor.
11. The composition according to Claim 1, wherein a protease cleavage site is present between said ligand and said polysaccharide binding domain.
12. The composition according to Claim 1, wherein said biocompatible solid phase is cellulose acetate, chitin or chitosan.
13. A composition comprising:
a complex comprising a viable growth factor responsive cell diffusively bound to a growth factor diffusively immobilized via a cellulose binding domain on a biocompatible support which comprises cellulose.
14. The composition according to Claim 13, wherein said growth factor is selected from the group consisting of stem cell factor, an insulin-like growth factor, EGF, FGF, an angiogenesis factor, a blood forming factor, FLT 3 ligand, steel factor, IL-2, IL-3, IL-6, GCSF, GMCSF, MCSF, EPO, a nerve cell growth factor, and a chondrogenic growth factor.
15. The composition according to Claim 13, wherein said growth factor responsive cell is selected from the group consisting of a hematopoietic cell, a fibroblast, an epithelial cell, a chondrocyte, and a nerve cell.
16. The composition according to Claim 13, wherein said biocompatible support is in a form selected from the group consisting of a membrane, an implantable device, an extracorporeal device, a plurality of beads, a powder, a sponge, and a wound dressing.
17. The composition according to Claim 16, wherein said beads are microbeads.
18. The composition according to Claim 16, wherein said wound dressing is an external wound dressing.

19. The composition according to Claim 16, wherein said biocompatible solid surface is biodegradable.
- 5 20. The composition according to Claim 19, wherein said biocompatible solid surface comprises an oxidized cellulase.
21. The composition according to Claim 13, wherein said cellulose is selected from the group consisting of plasticized cellulose, cellulose acetate,
10 ethylhydroxyethylcellulose.
22. The composition according to Claim 13, wherein said cellulose is a cellulose derivative.
- 15 23. A method for growth factor receptor mediated activation of intracellular tyrosine kinase in a growth factor dependent cell, said method comprising:
contacting said growth factor dependent cell with a composition comprising a ligand for said growth factor receptor diffusibly immobilized on a biocompatible solid phase via a polysaccharide binding domain whereby said growth factor receptor binds
20 to said ligand to form a growth factor receptor-ligand complex which diffuses in the membrane of said cell to dimerize with an adjacent growth factor receptor-ligand complex, whereby intracellular tyrosine kinase in said cell is activated.
24. The method according to Claim 23, wherein said ligand is a small molecule
25 mimetic of a growth factor.
25. A method for enhancing the rate of division of growth factor dependent cells, said method comprising:
contacting a culture containing a plurality of growth factor dependent cells
30 with a biocompatible support to which a biologically active growth factor is diffusively bound via a polypeptide which binds to a polysaccharide, whereby said cells bind to said growth factor via a cell surface receptor for said growth factor to

form a growth factor receptor ligand complex which diffuses in a membrane of said cell to dimerize with a second growth factor receptor-ligand complex, wherein the amount of said biologically active growth factor is sufficient to transiently activate said cell surface receptor whereby one or more growth factor receptor mediated
5 signals in said cell are activated, whereby the rate of division of said cells is enhanced.

26. The method according to Claim 25, wherein a protease cleavage site is present between said growth factor and said polypeptide.

10 27. The method according to Claim 25, further comprising:
cleaving said complex from said biocompatible support when said culture has expanded to a sufficient size.

28. The method according to Claim 25, wherein said biocompatible support is in a
15 form selected from the group consisting of a glass culture vessel, a tissue culture dish, a petri dish, a multiwell plate, a membrane, an implantable device, a sponge, a plurality of beads, a plurality of microbeads, a hollow fiber, a roller bottle or a culture flask.

20 29. The method according to Claim 25, wherein said biocompatible support is cellulose acetate.

30. A method for obtaining hematopoietic cells for autologous implantation, said method comprising:

25 growing a culture containing a plurality of hematopoietic cells obtained from an animal on a biocompatible support diffusively coated with a growth factor via a polypeptide which binds to a polysaccharide, whereby said cells bind to said growth factor via a cell surface receptor said growth factor to form growth factor receptor ligand complex, wherein the amount of said biologically active growth factor is
30 sufficient to transiently activate said growth factor receptor, whereby growth of said cells is stimulated; and

harvesting said cells when said culture has expanded to a sufficient size,
whereby
hematopoietic stem cells for autologous implantation are obtained.

5 31. The method according to Claim 30, wherein said growth factor is EPO or steel factor.

32. The method according to Claim 30, wherein said hematopoietic cells are from bone marrow or peripheral blood.

10

33. A method for stimulating hematopoietic cells from bone marrow or peripheral blood to differentiate, said method comprising:
growing a culture containing a plurality of bone marrow cells on a biocompatible support diffusively coated with one or more growth factors selected
15 from the group consisting of FLT ligand, steel factor, GCSF, GMCSF, MCSF, and IL-3, wherein the amount of said one or more growth factors is sufficient for prolonged activation of growth factor receptors on said hematopoietic cells, whereby said hematopoietic cells are stimulated to differentiate.

20 34. A method for presenting a biologically active polypeptide immobilized on a solid support in a biologically preferred conformation for binding to a receptor for said polypeptide, said method comprising:

binding said growth factor to said solid support via a polysaccharide binding domain which diffuses on said solid support.

25

35. A method for modulating surface presentation of receptor molecules on a cell, said method comprising:

contacting a cell containing biologically active cell surface receptors for a polypeptide with a biocompatible support to which a biologically active growth factor
30 is diffusively bound via a polypeptide which binds to a polysaccharide, whereby said cells bind to said growth factor via a cell surface receptor for said growth factor to form a growth factor receptor ligand complex which diffuses in the membrane of said

cell to dimerize with a second growth factor receptor-ligand complex whereby one or more growth factor receptor mediated signals in said cell are activated, whereby growth of said cell is modulated.

- 5 36. A method for rescuing bone marrow cells from a cell population contaminated with cancer cells, said method comprising:

contacting said cell population with a composition comprising steel factor immobilized on a solid support via a cleavable linkage in an amount sufficient for prolonged activation of a steel factor receptor on said bone marrow cells which bind
10 to said steel factor so that growth of said bone marrow cells is inhibited in tandem with a cytotoxic agent in an amount sufficient to kill said cancer cells; and

removing said bone marrow cells from said solid support so that said steel factor is internalized by said bone marrow cells and said cells are stimulated to divide, whereby said bone marrow cells are rescued from said cell population.

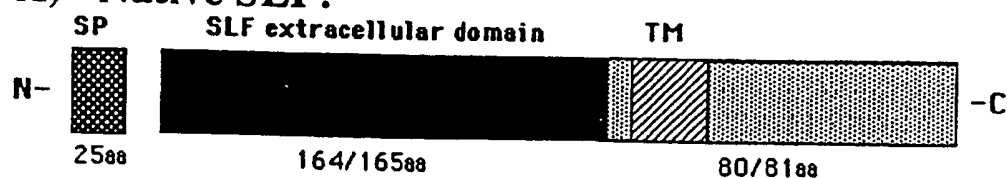
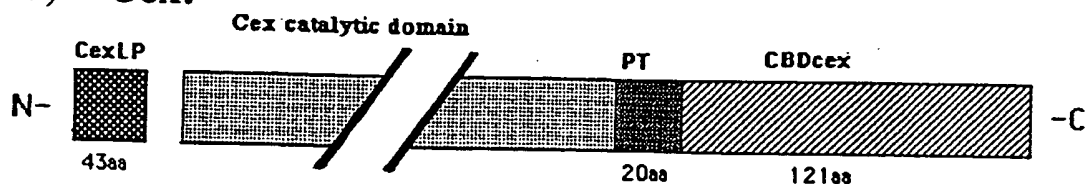
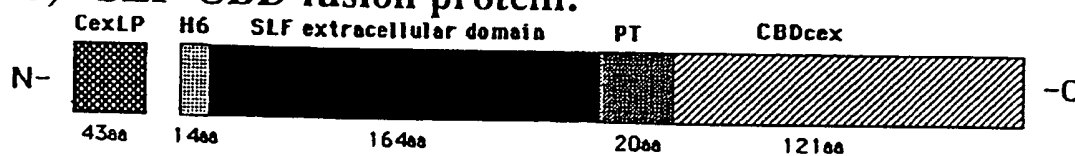
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37. A composition for autologous implantation comprising:

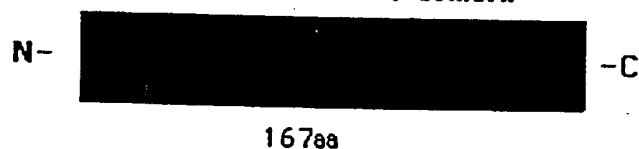
a plurality of hematopoietic stem cells obtained according to the method of Claim 30.

38. A method for modifying the rate of division of growth factor dependent cells, said
20 method comprising:

contacting a culture containing a plurality of growth factor dependent cells with a biocompatible support to which a biologically active growth factor is bound via a polypeptide which binds to a polysaccharide, whereby said cells bind to said growth factor via a cell surface receptor for said growth factor to form a growth factor
25 receptor ligand complex.

A) Native SLF:**B) Cex:** 27.6kDa (processed and non-glycosylated)**C) SLF-CBD fusion protein:**

(HHHHHHIEGRARKEN)

34.1kDa (processed)
(PPVASIEGR)***D) Recombinant SLF control:**
SLF extracellular domain

18.6 kDa (non-glycosylated)

FIG. 1

XbaI g10 Leader NcoI EcoRI MCS HindIII
 M E F E L G T R G S S R V D L Q A C K L
 AtctagaAATAATTGTGTTAACTTTAAGAGGAGATATATccatggaattcGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCaagctt
 TagatcCtTATTAAACAATTCCTcctCTATATAGgtacctaagCTCCACCCATGGGCCCTTAGGAGATCTCAGCTGGACGTCCCGTACGttcgaa

FIG. 2A

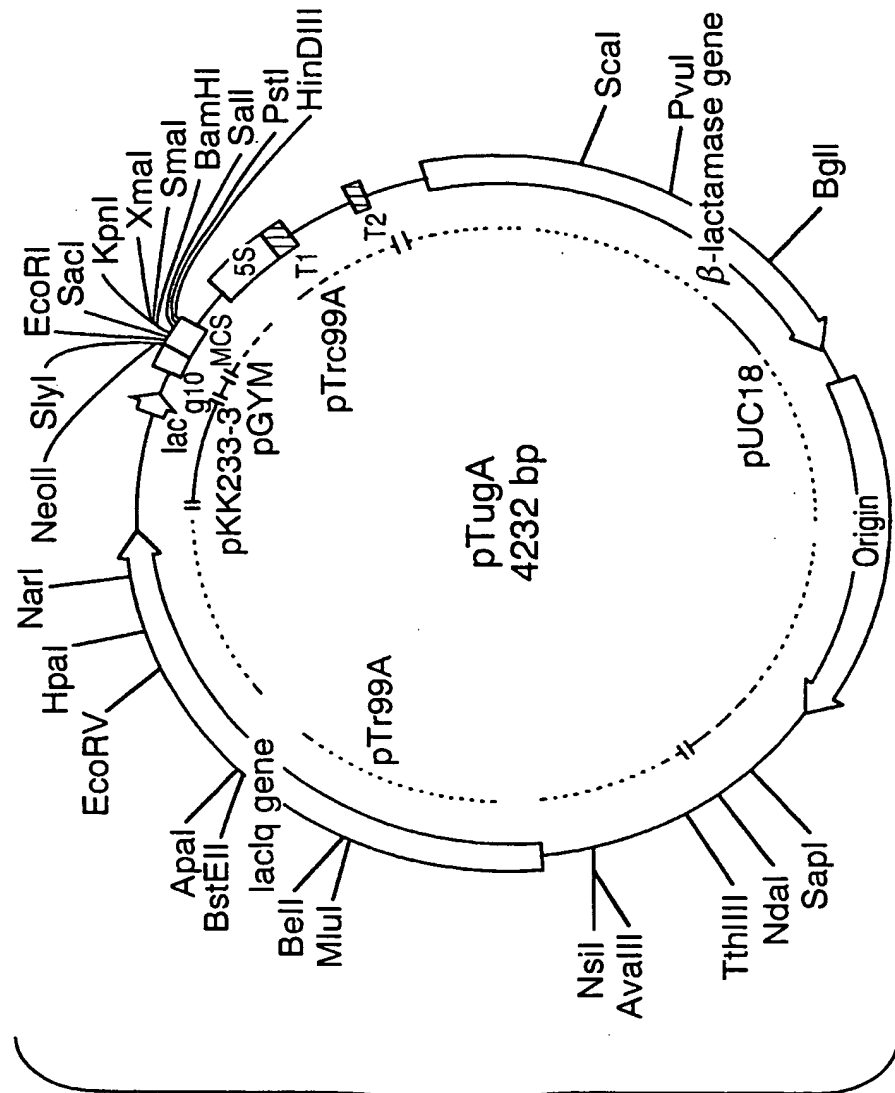


FIG. 2B

g10 Leader SacI M S T R (CBD) P T P I E G R P E F Q L G T R G S S R V D L Q A C K L HindIII
rba ..TTTAACTTTAAGAaggagctcCTTCATGTCCACCGC.....CCACGGCGATCGAGGCGaggctgaattccAGCTGGTACCGGGGATCCTCTAGACTCGACCTGCAGGCATGCaagctt
..AAATTGAAATTCTtctctgagGAACTACAGGTGGGC.....GGGTGGGCTAGTCCCGctgtgacttaagGTCGAGCCATGGGCCCCCTAGGAGATCTCAGCTGGAGCTCCGTACGgttcgaa

FIG. 3A

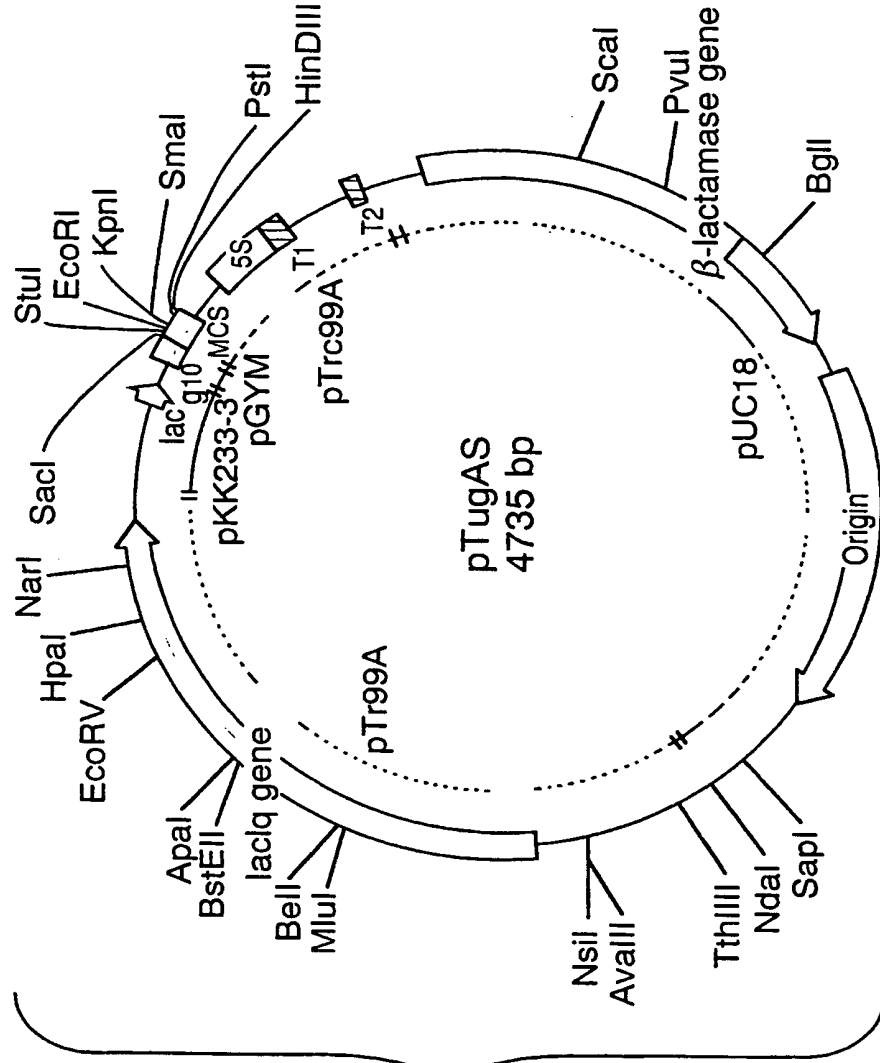


FIG. 3B

4/34

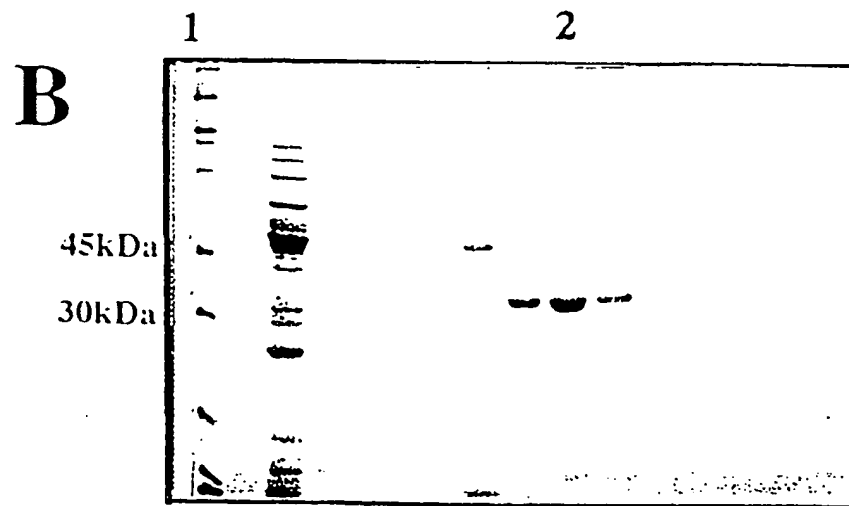
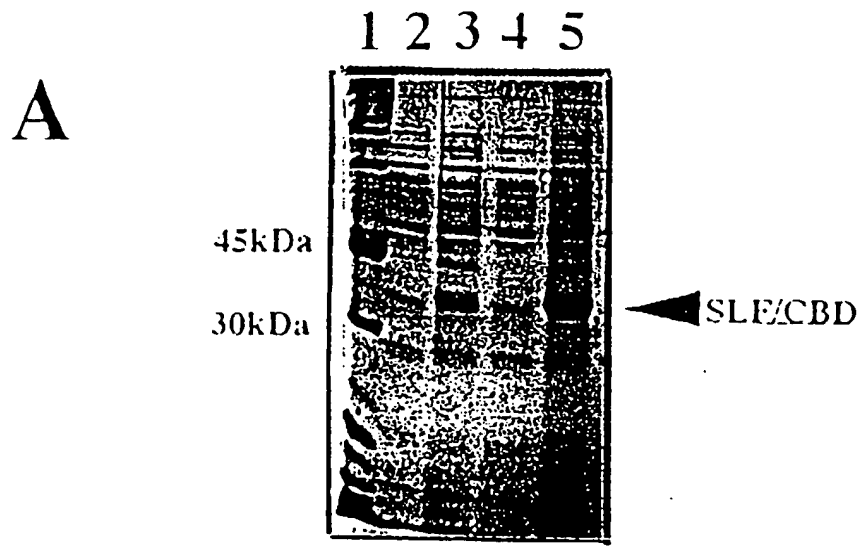


Fig. 4

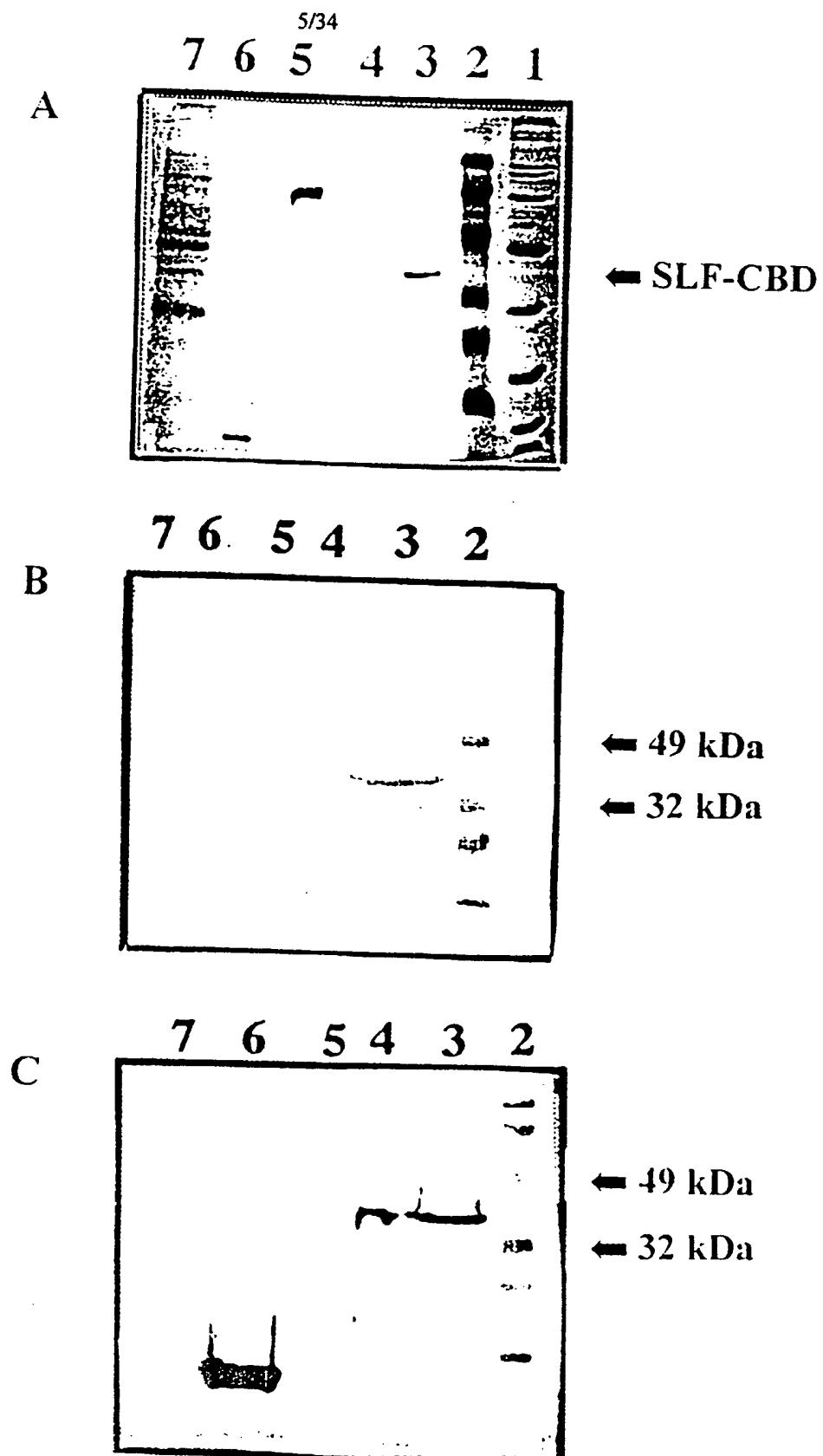


Fig. 5

6/34

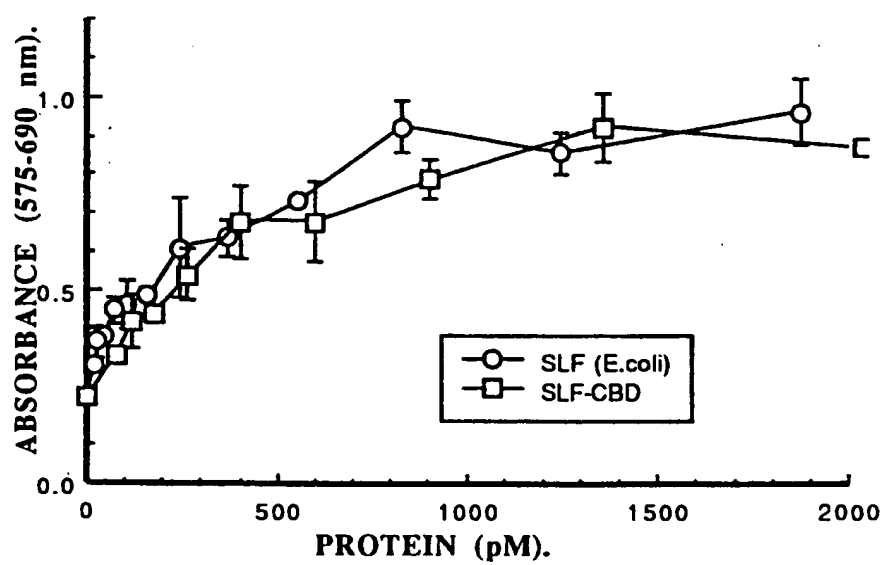


FIG. 6A

7/34

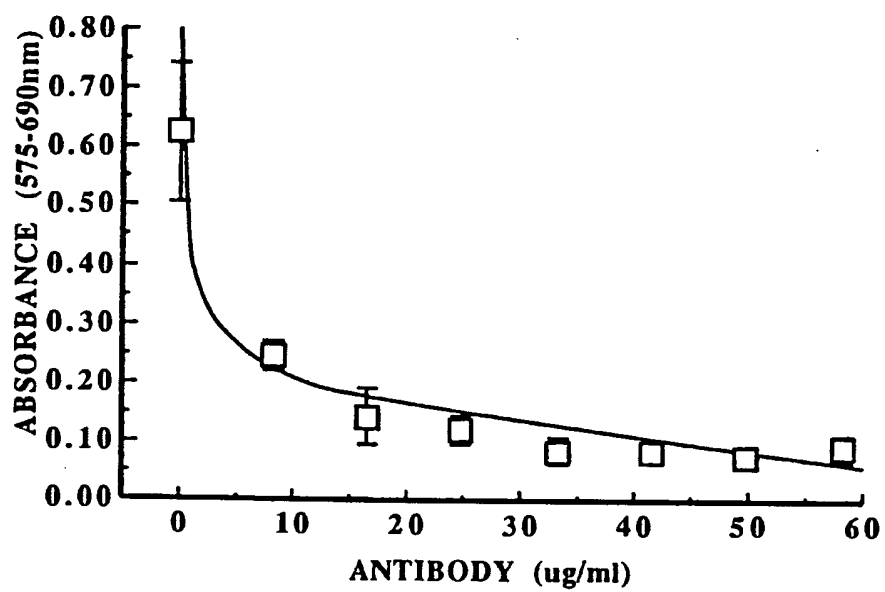


FIG. 6B

8/34

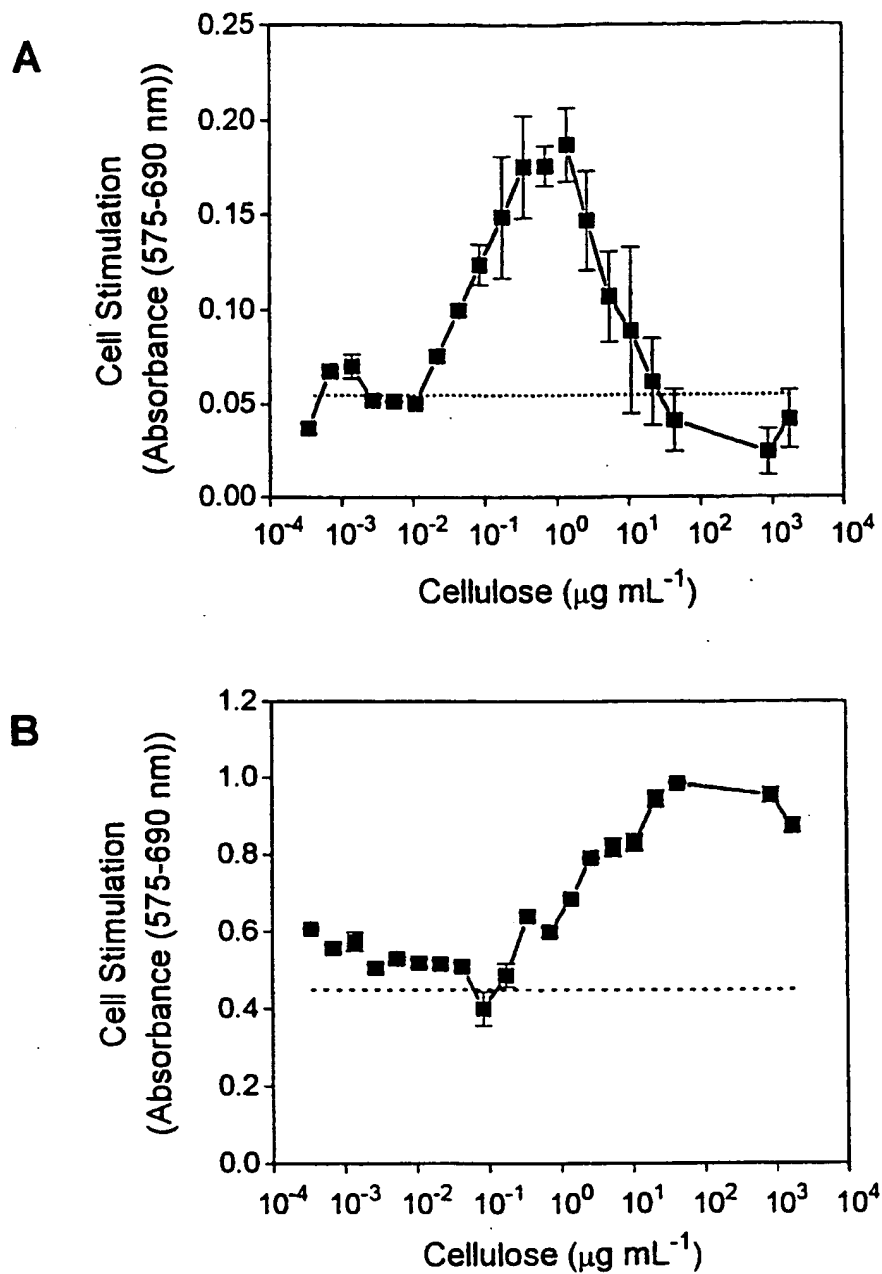


FIG. 7

9/34

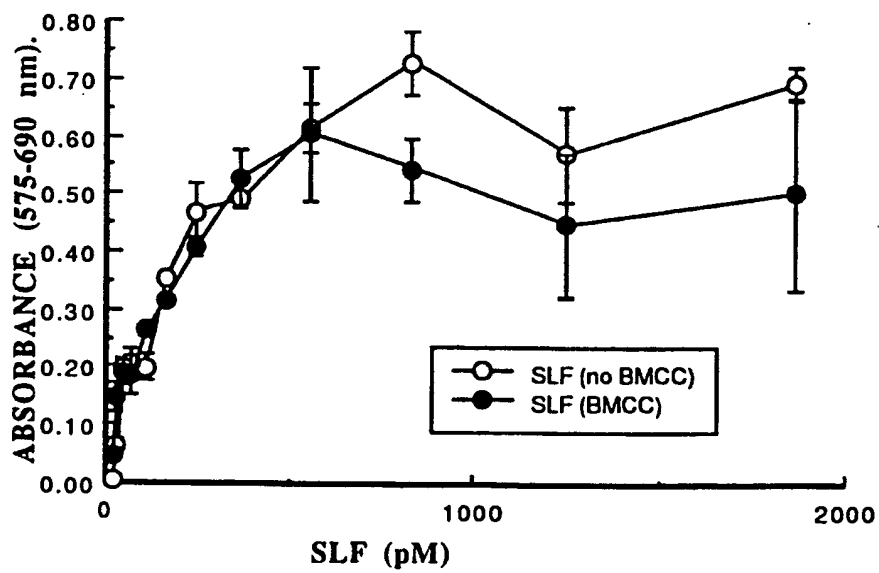


FIG. 8A

10/34

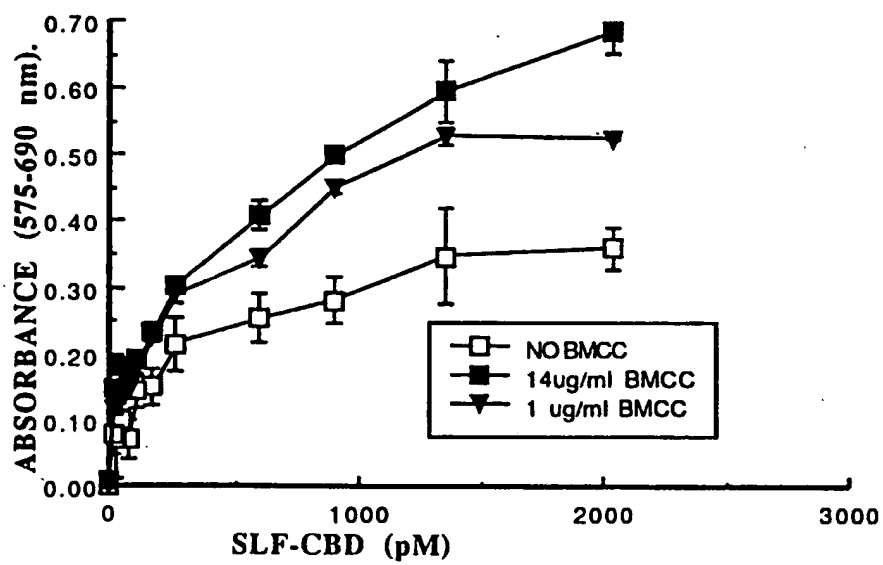


FIG. 8B

11/34

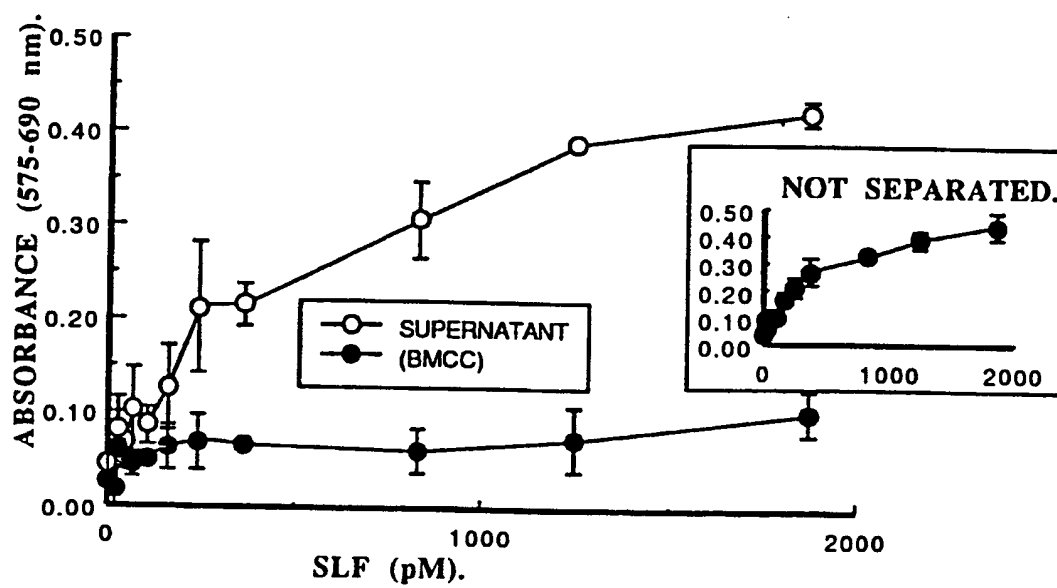


FIG. 9A

12/34

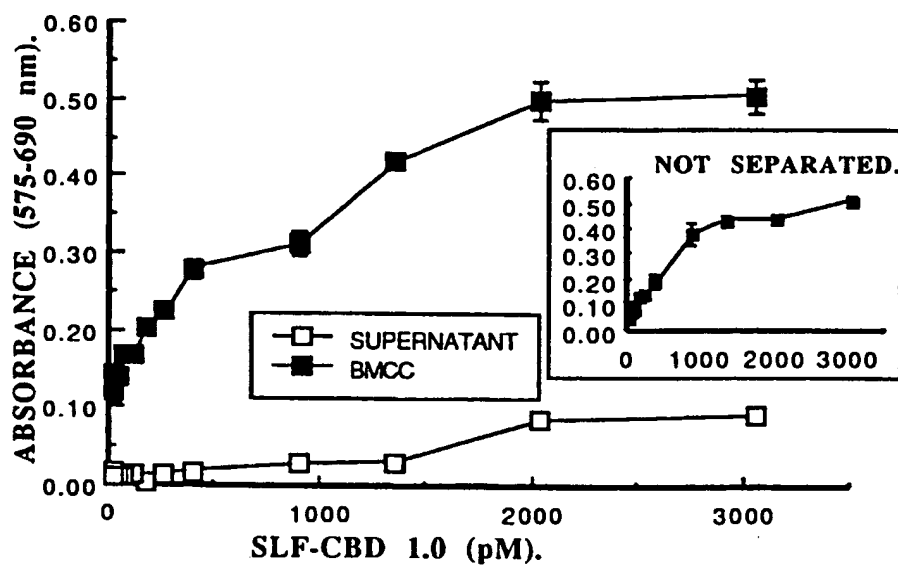


FIG. 9B

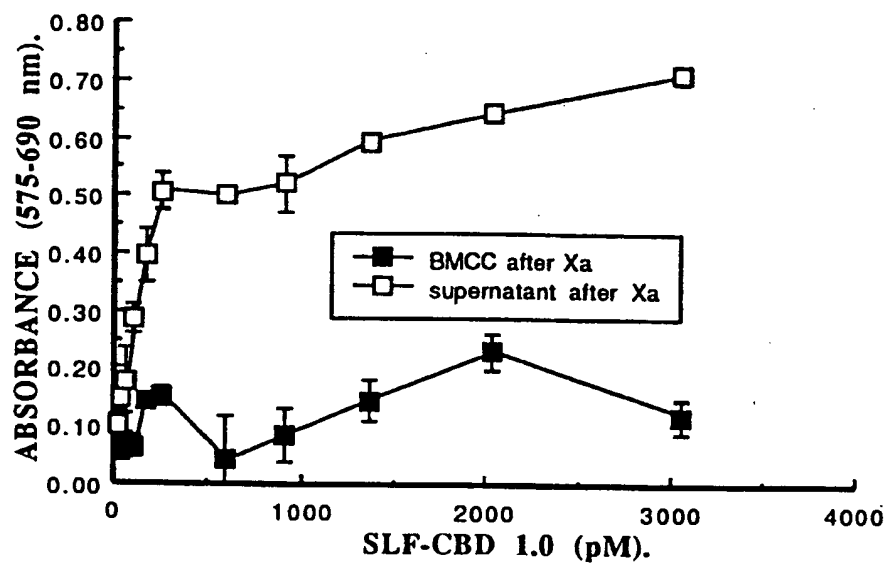


FIG. 9C

14/34

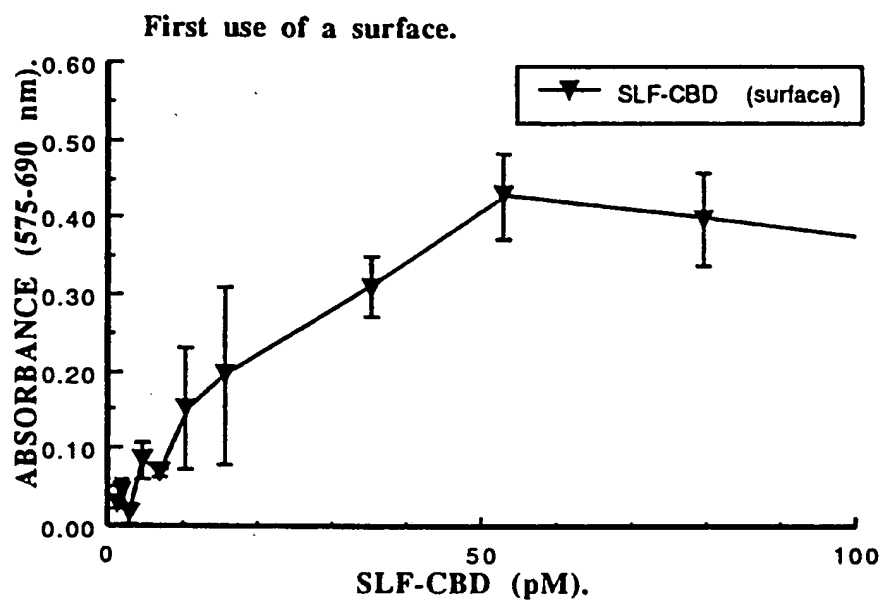


FIG. 10A

15/34

Third use of surfaces.

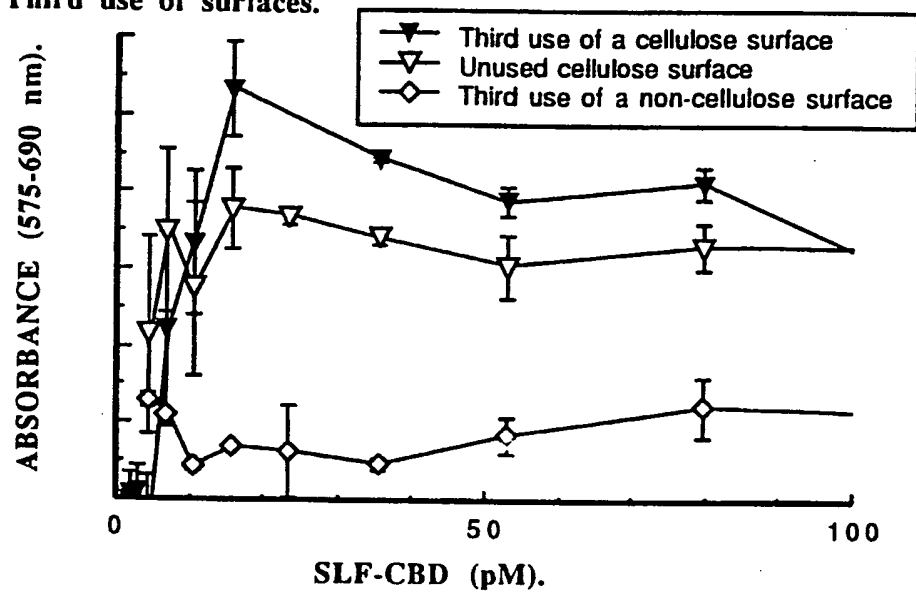


FIG. 10B

16/34

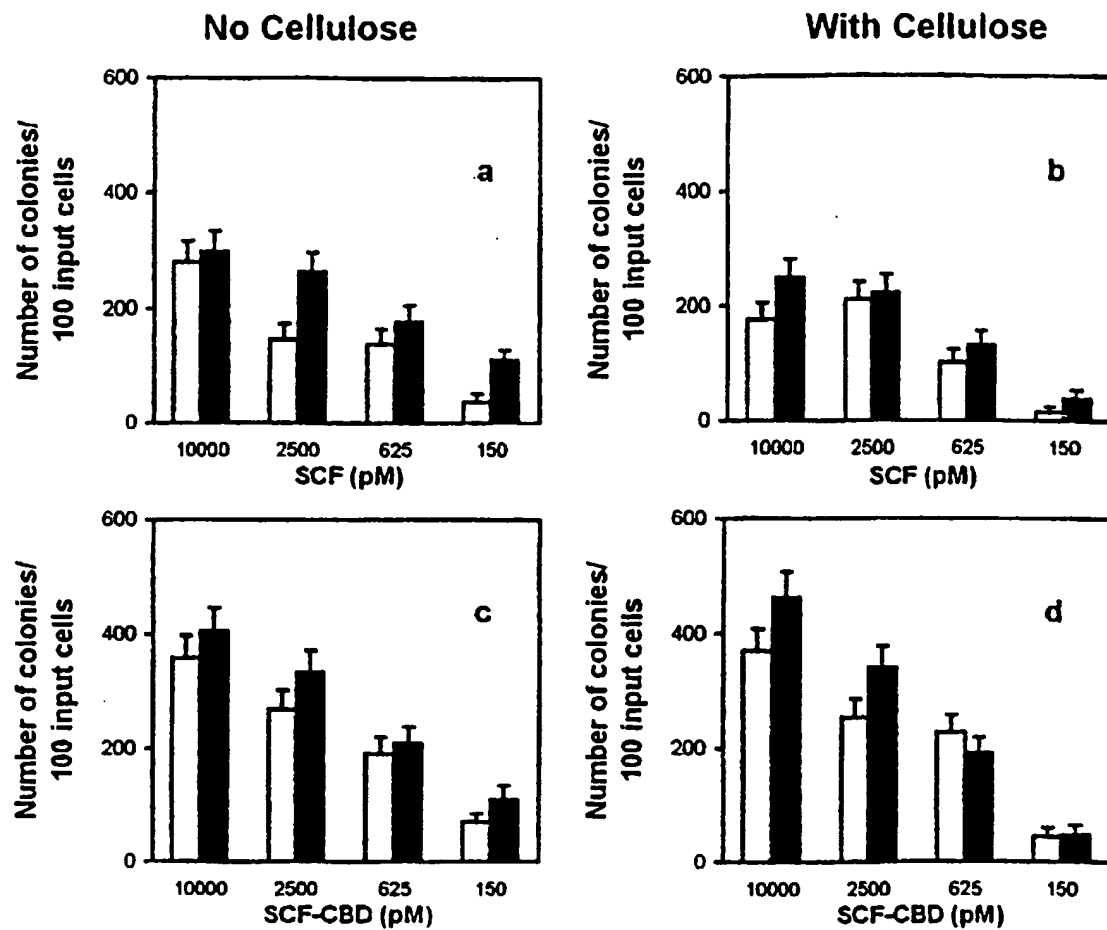


Fig. 11A

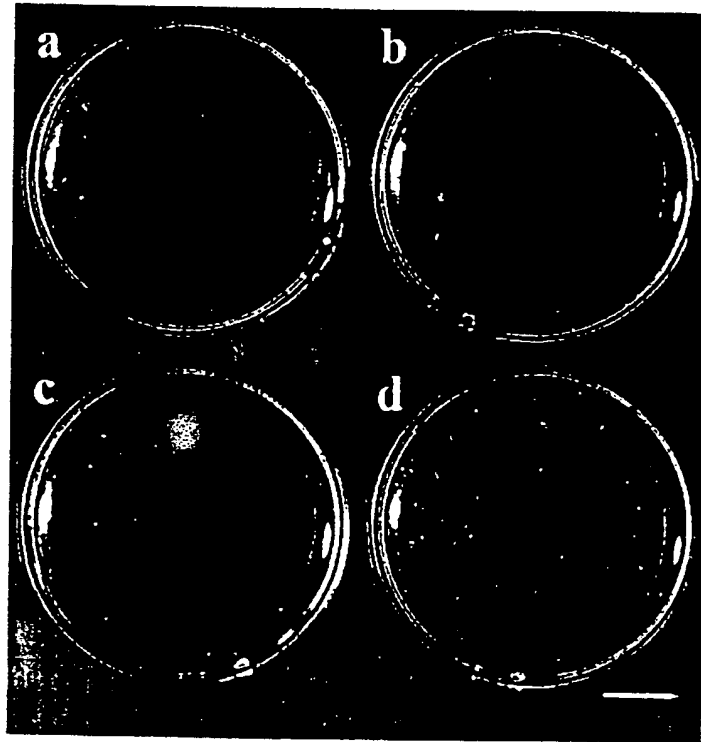


Fig. 11B

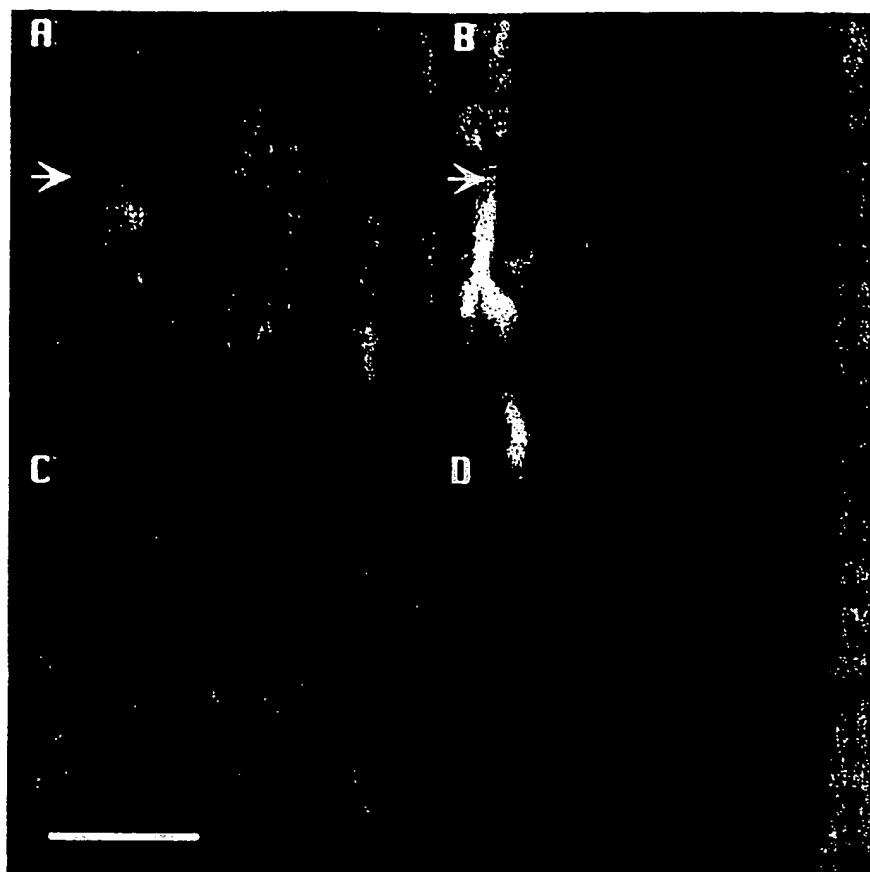


Fig. 12

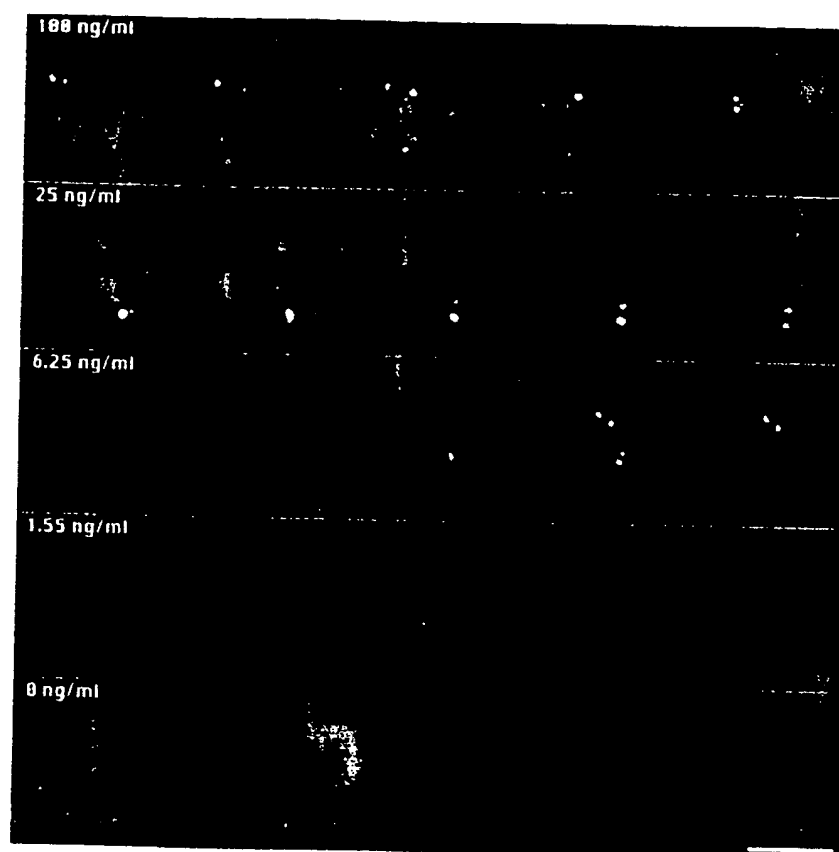


Fig. 13

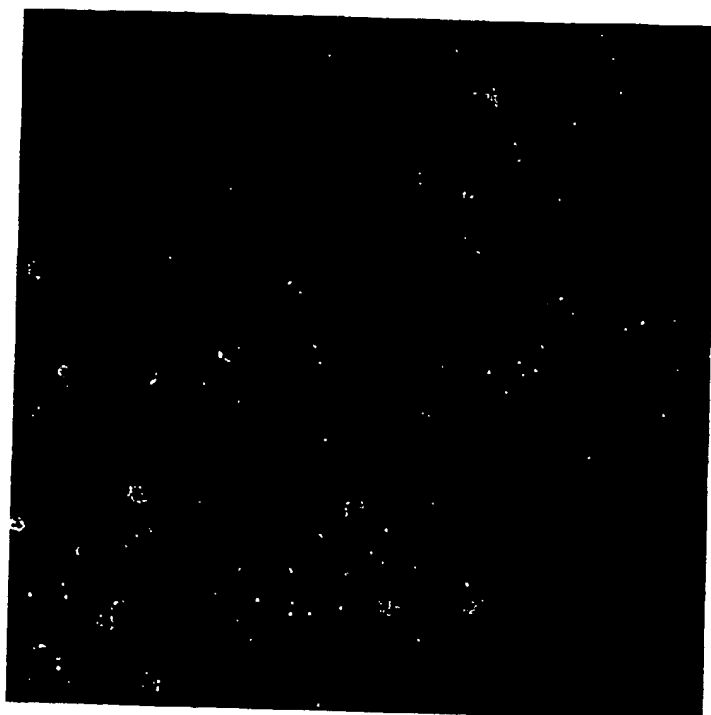


Fig. 14

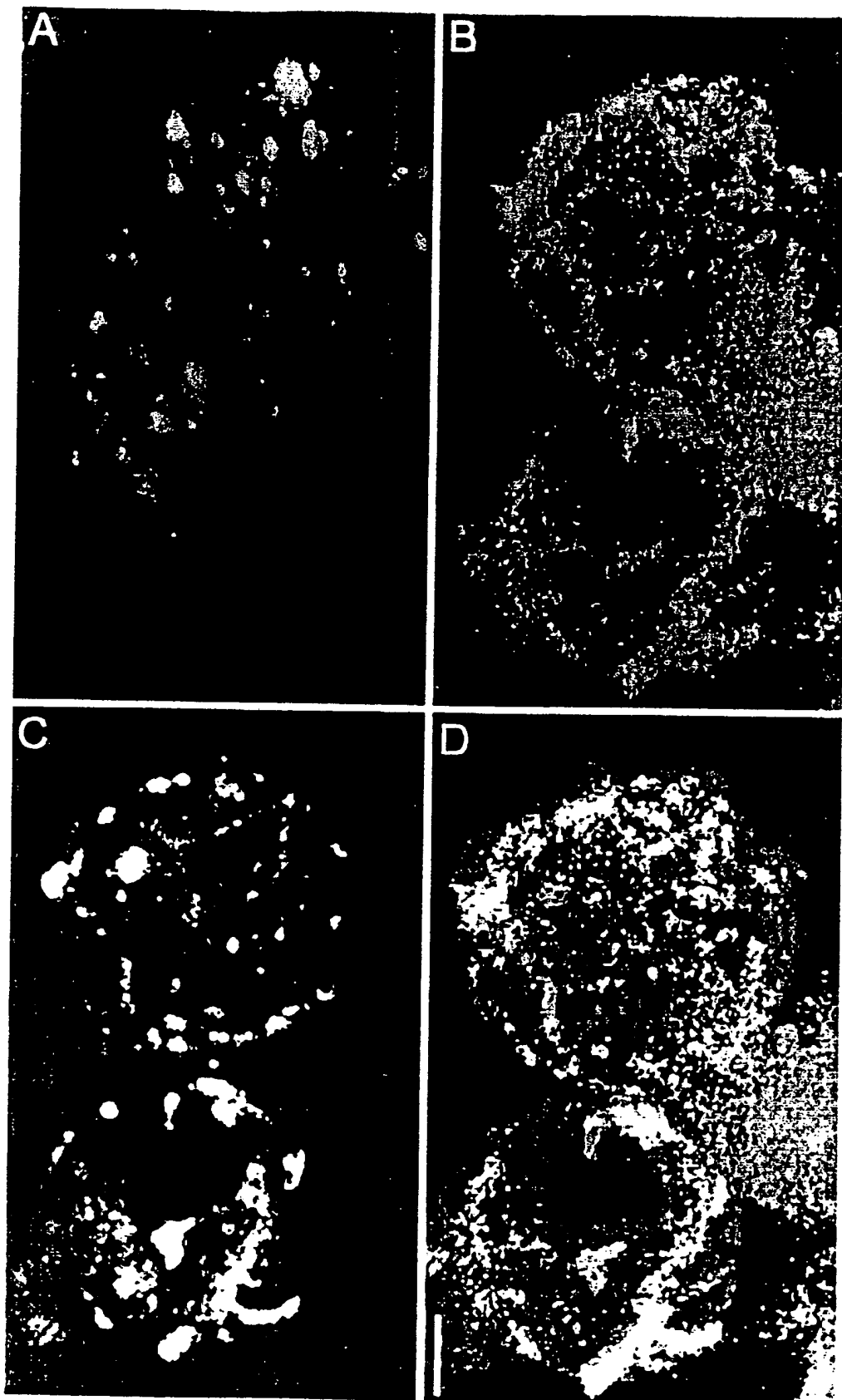


Fig. 15

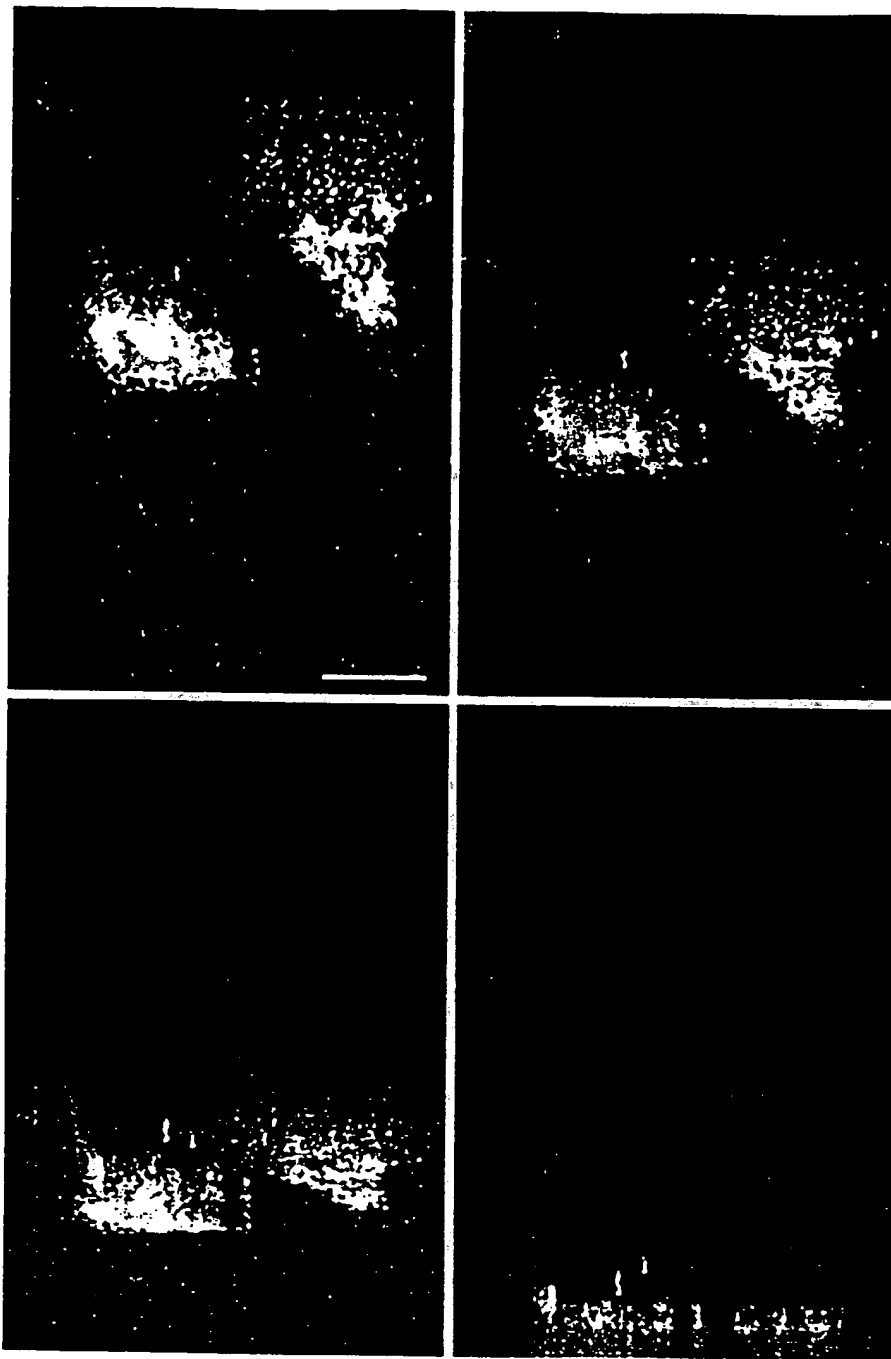


Fig. 16

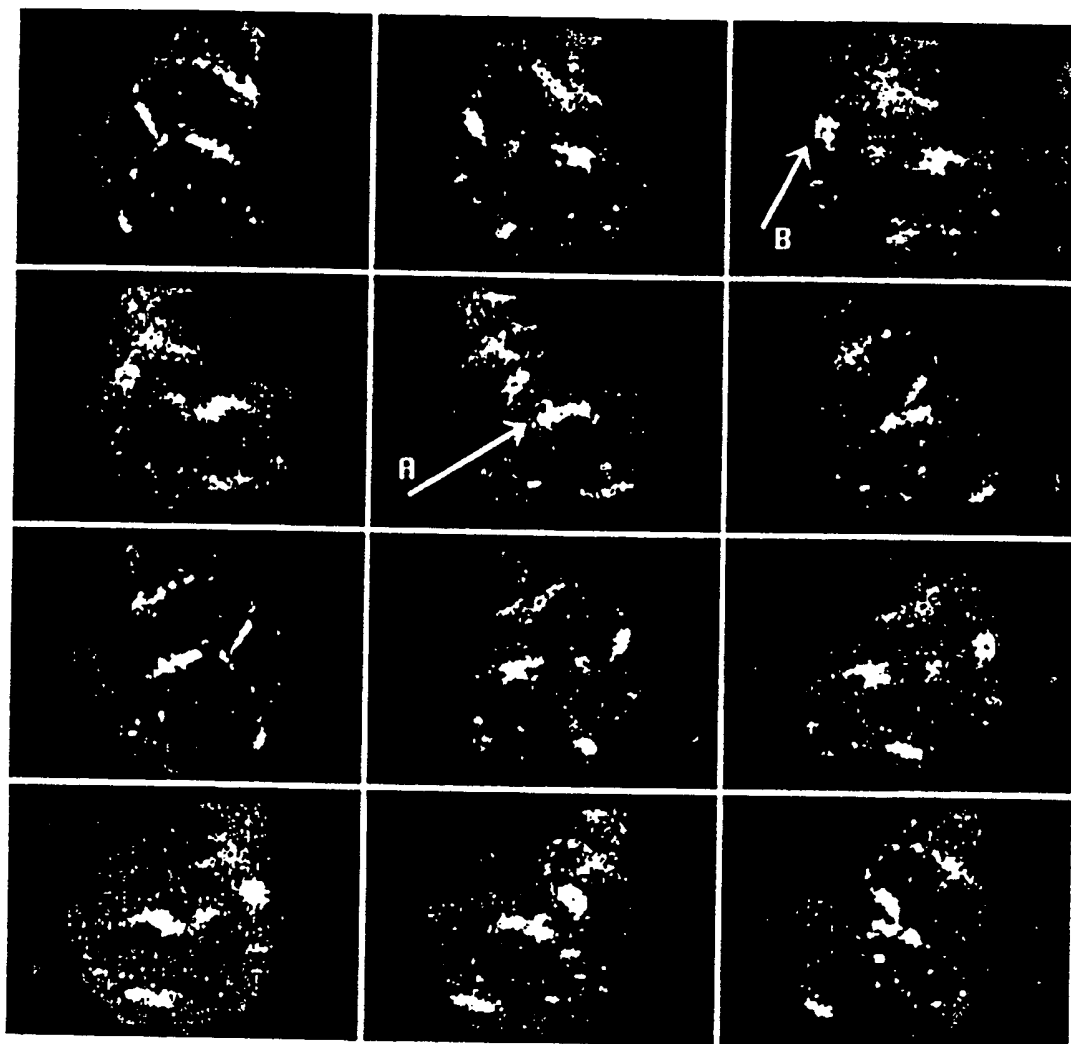


Fig. 17

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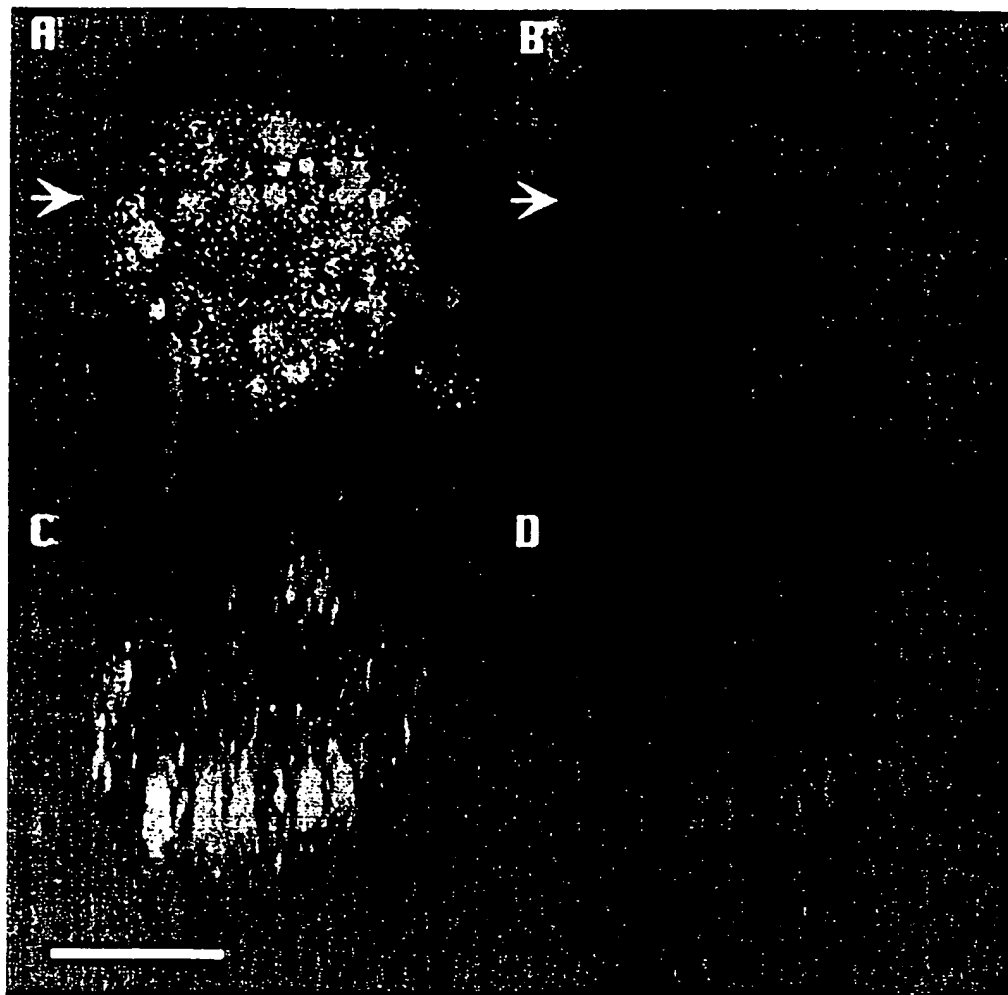


Fig. 18



FIG. 19

26/34

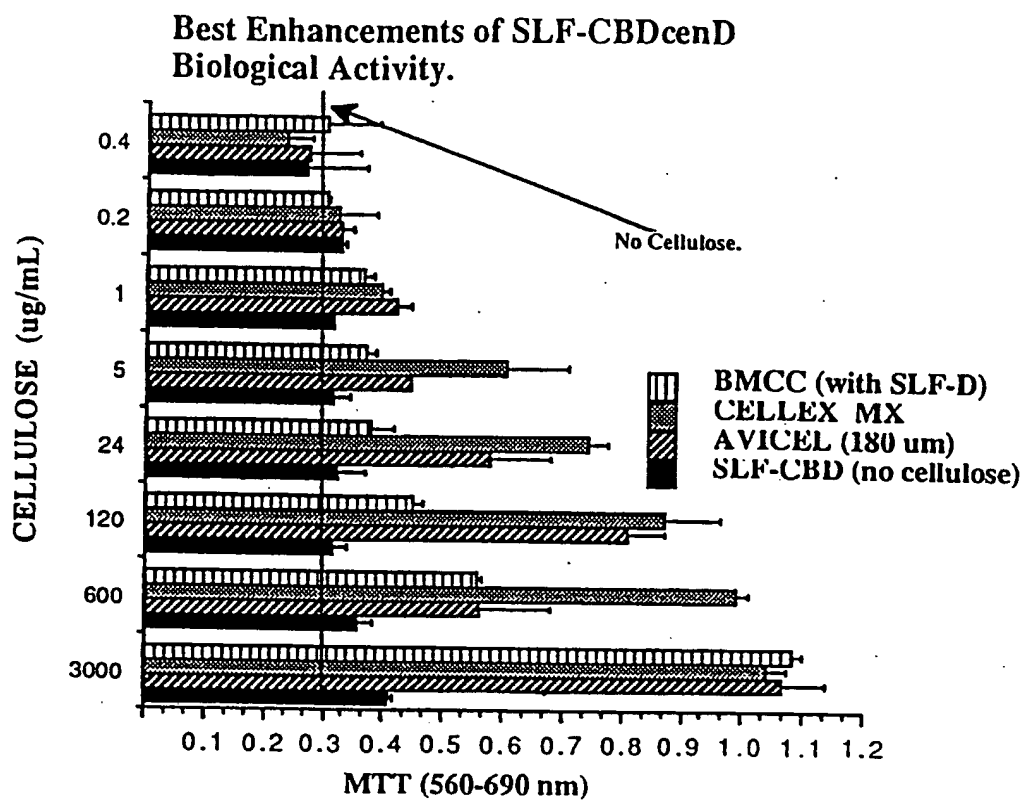


FIG. 20

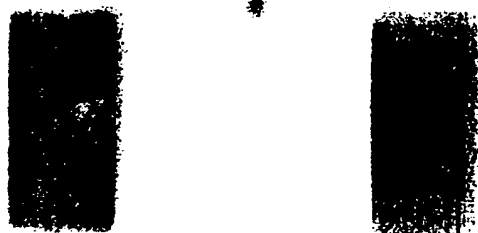


Fig. 21

28/34

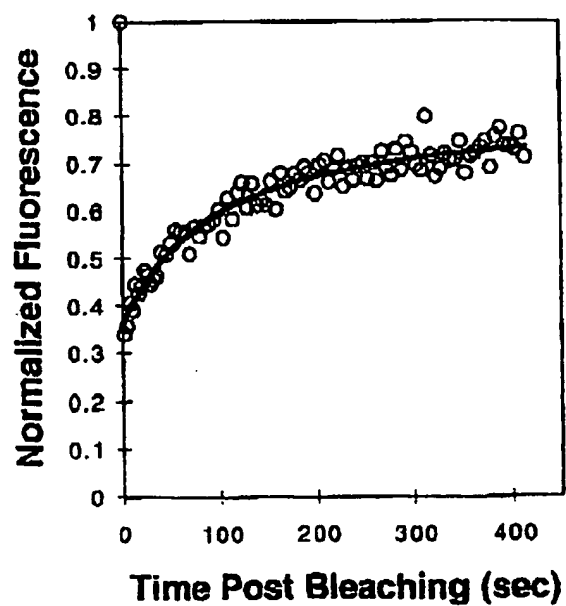
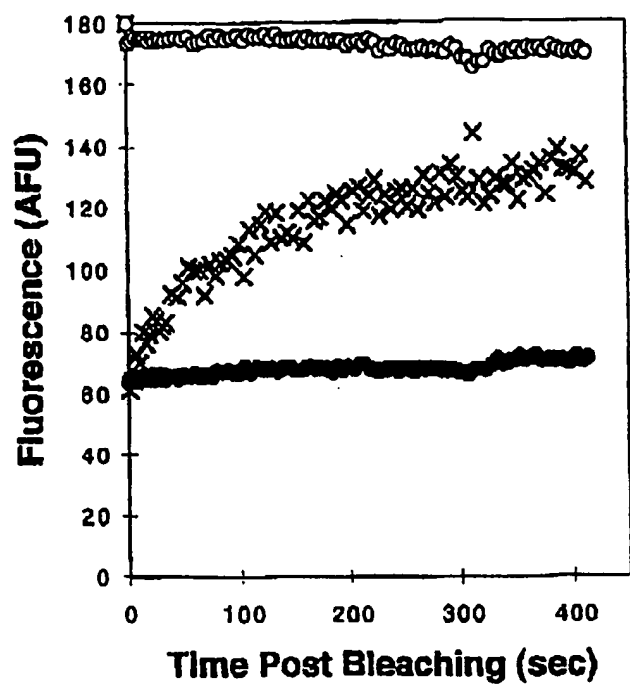


FIG. 22

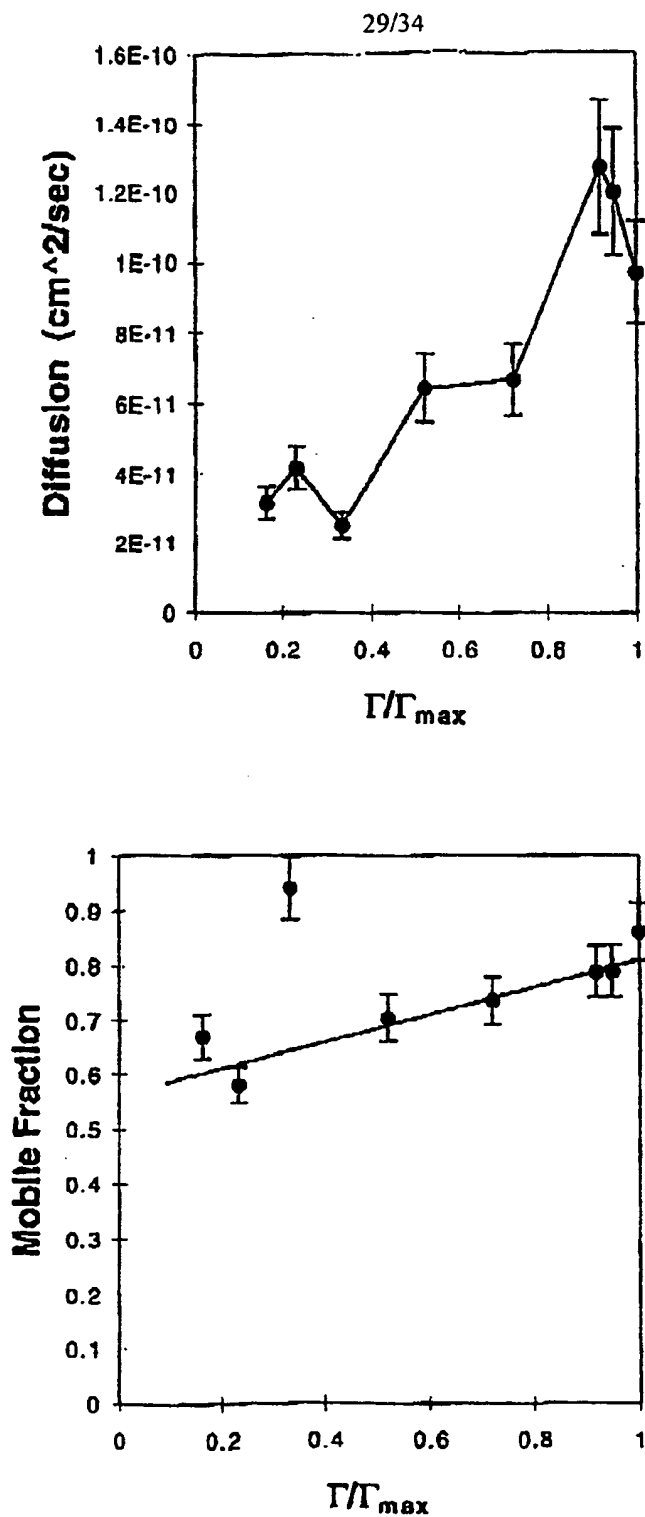


FIG. 23

30/34

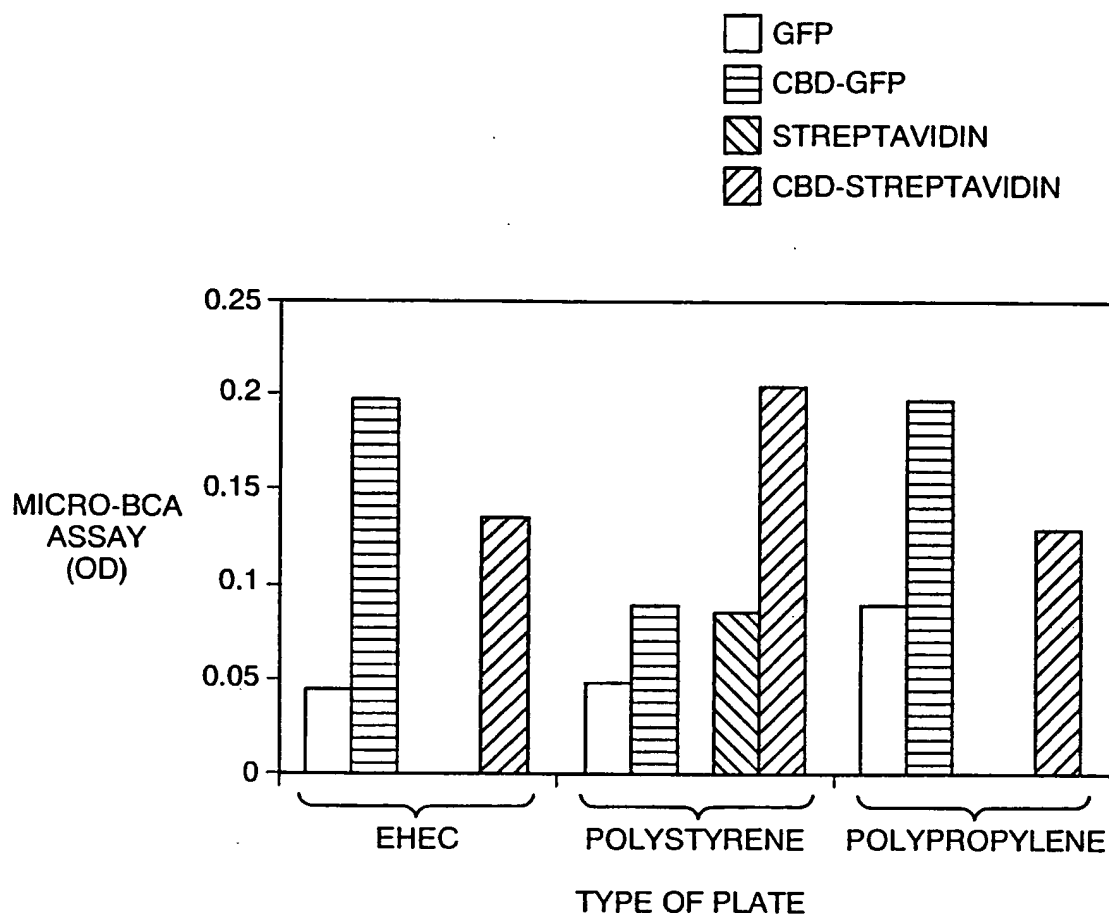


FIG. 24

31/34

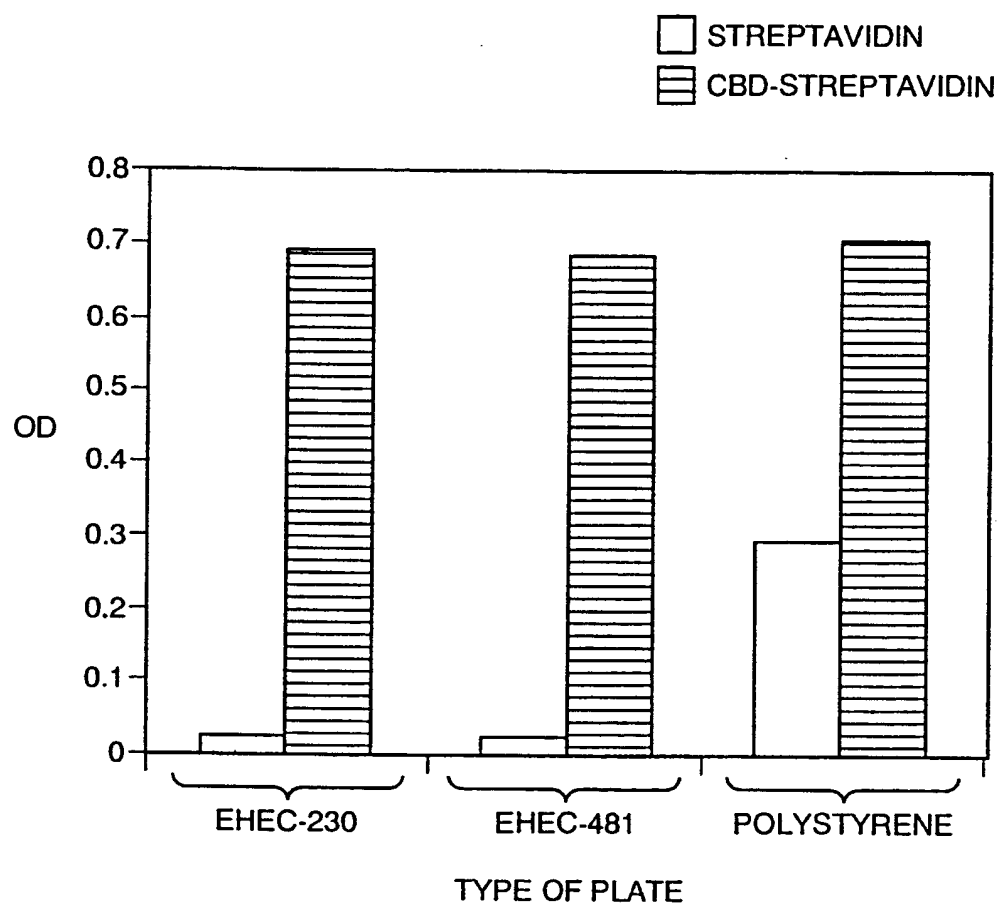


FIG. 25

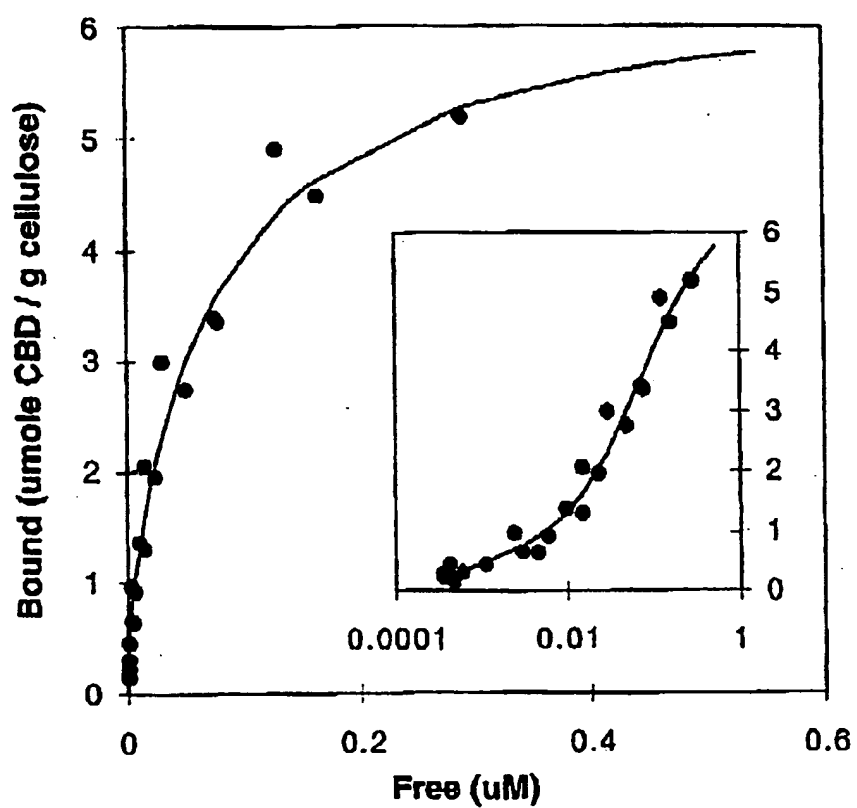


FIG. 26

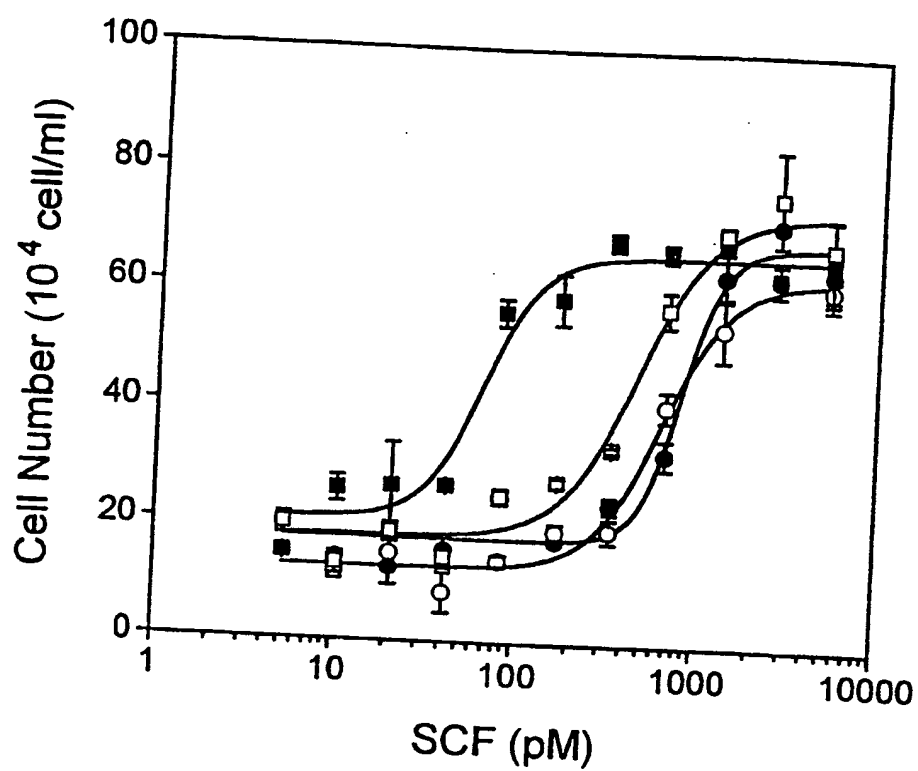


FIG. 27

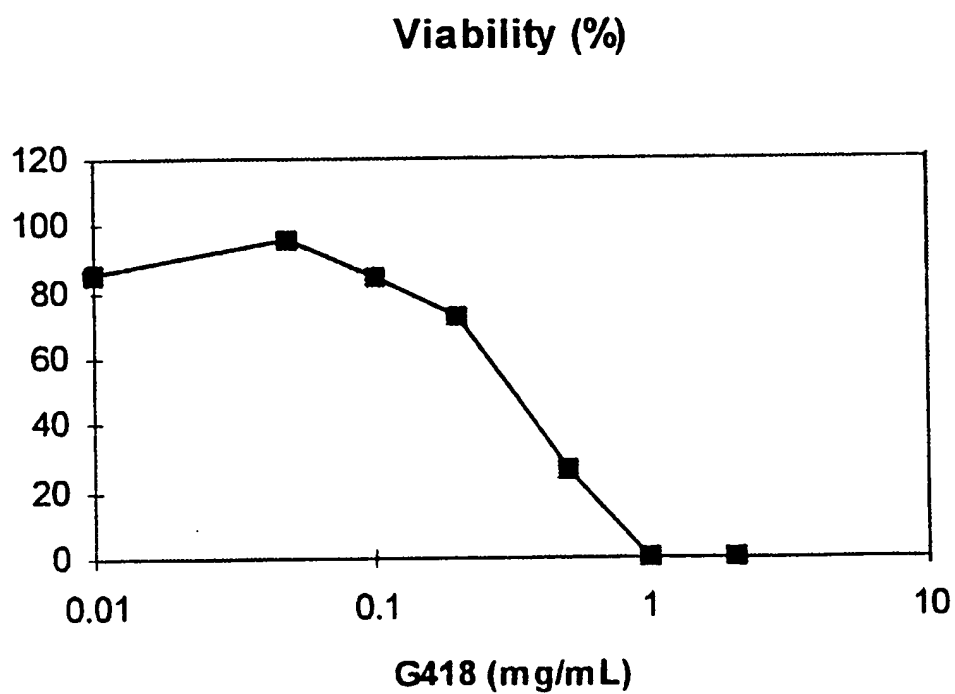


Fig.28

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